

Original Article

Cite this article: de Souza VG, Bandeira LB, Souza NCS, Taboga SR, Martins TMM, and Silva Perez AP. Prenatal and pubertal exposure to 17 α -ethinylestradiol disrupts folliculogenesis and promotes morphophysiological changes in ovaries of old gerbils (*Meriones unguiculatus*). *Journal of Developmental Origins of Health and Disease* doi: [10.1017/S2040174421000040](https://doi.org/10.1017/S2040174421000040)

Received: 29 September 2020

Revised: 13 January 2021

Accepted: 19 January 2021


Keywords:

Gerbillinae; ovary; contraceptive agents; endocrine disruptors

Address for correspondence:

Dr. Ana Paula Silva Perez, Institute of Health Sciences, Medicine Course – Federal University of Goiás – UFG, Jataí, Goiás, BR 364, Km 194, number 3800, Class of Teachers II, number 26, Jatobá campus – University City, Jataí, GO, Brazil. Email: paulabio_perez@ufg.br

Prenatal and pubertal exposure to 17 α -ethinylestradiol disrupts folliculogenesis and promotes morphophysiological changes in ovaries of old gerbils (*Meriones unguiculatus*)

Vinícius Gonçalves de Souza¹, Laura Borges Bandeira¹,
Nátaly Caroline Silva e Souza¹, Sebastião Roberto Taboga^{2,3},
Tracy Martina Marques Martins¹ and Ana Paula Silva Perez⁴ 

¹Institute of Health Sciences, Medicine Course, Federal University of Jataí, UFJ, Jataí, Goiás 75801-615, Brazil;

²Department of Biology, Laboratory of Microscopy and Microanalysis, São Paulo State University – UNESP, São

José do Rio Preto, São Paulo 15054-000, Brazil; ³Department of Structural and Functional Biology, State

University of Campinas – UNICAMP, Campinas, São Paulo 13083-970, Brazil and ⁴Institute of Health Sciences,

Medicine Course and Graduate Program of Animal Bioscience, Federal University of Jataí, UFJ, Jataí, Goiás

75801-615, Brazil

Abstract

17 α -Ethinylestradiol is an endocrine-disrupting chemical that make up most contraceptive pills and can be found in the environment. Exposure to ethinylestradiol in different development periods may promote changes in morphophysiological parameters of reproductive and endocrine organs. Considering that the effects of low doses (15 μ g/kg/day) of ethinylestradiol in ovaries from 12-month-old female gerbils (*Meriones unguiculatus*) were investigated. Four experimental groups used were control (without treatment), EE/PRE (treated from the 18th to the 22nd gestational day), EE/PUB (treated from the 42nd to the 49th day of life), and EE/PRE-PUB (treated in the both periods). The animals were euthanized at 12 months. Testosterone and 17 β -estradiol levels were measured. The ovaries were stained with Hematoxylin and Eosin, Periodic Acid Schiff, and Gomori's Trichome. The follicles, corpus luteum, interstitial gland, lipofuscin, ovarian epithelium, and tunica albuginea were analyzed. Estradiol was higher in EE/PRE and EE/PUB groups, while testosterone was higher only in EE/PUB group. The main changes in follicle count occurred in EE/PUB and EE/PRE-PUB groups, with higher primordial follicle count and lower maturation of follicles. The corpus luteum was more evident in EE/PRE group. No differences were found in atretic follicles count. A higher area occupied by interstitial gland cells and lipofuscin deposit in these cells was noted in EE/PUB and EE/PRE-PUB groups. Higher epithelium height and thicker tunic albuginea were showed in treated groups. These results suggest that exposure to doses of EE2 in prenatal and pubertal periods of the development leads to morphological changes in senile ovaries.

Introduction

Endocrine-disrupting chemicals (EDCs) are exogenous compounds that can disrupt the morphology and function of endocrine and reproductive systems of animals and humans.¹ Many EDCs have the potential to mimic, antagonize, or change the levels of endogenous hormones, such as androgens and estrogens.² 17 α -Ethinylestradiol (EE2), an example of endocrine disruptor, is a synthetic estrogen that makes up most contraceptive drugs. Moreover, this compound can be found in the environment, especially in water, in the ground, and in food.³

The prenatal, neonatal, and pubertal periods can be severely altered by EDCs because during these phases, changes in the serum levels of steroid hormones may impact the development of female reproductive organs.⁴ In rodents, the oocyte development occurs in the last half of fetal life and in the beginning of postnatal life by the rupture of oocyte nests and the formation of primordial follicles. During these phases, estrogens play an important role in the regulation of proliferation and apoptosis of follicle nests and stroma cells.⁵ Furthermore, during the reproductive life, these hormones are essential for ovary hormonal response and folliculogenesis by promoting proliferation of theca and granulosa cells and maturation of follicle.⁶

On the other hand, exposure to EE2 and other EDCs during developmental periods may impair the reproductive function of rodents by promoting changes in expression of the luteinizing hormone receptor gene, irregularities in the estrous cycle,³ changes in ovarian follicle development,⁷ and the hormonal profile of rodents.⁸

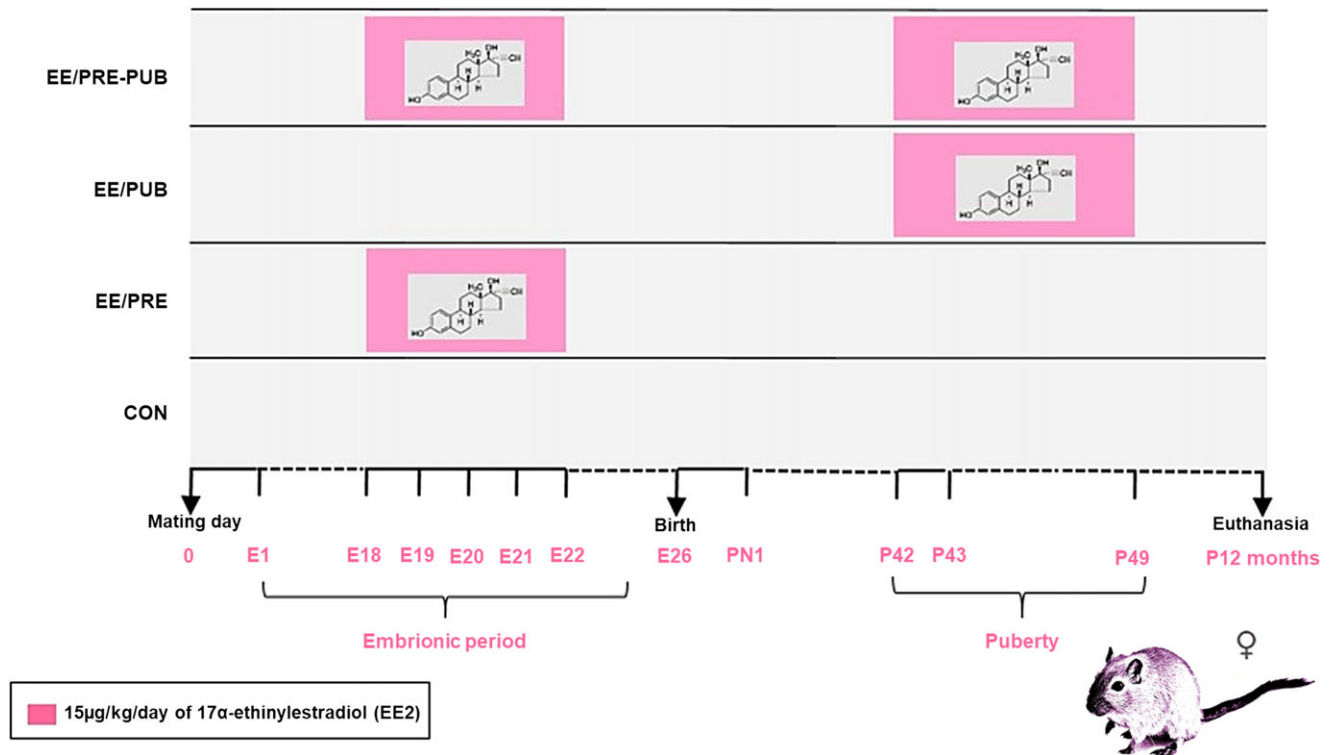


Fig. 1. Schematic representation of the experimental design. Pregnant females received 15 µg/kg/day of 17 α -ethinylestradiol (EE2) from the 18th to the 22nd gestational day. The pups of these females composed the prenatal group (EE/PRE). The pubertal group (EE/PUB) was composed of gerbils that received 15 µg/kg/day of EE2 between the 42nd and 49th days of postnatal life. In the prenatal and pubertal group (EE/PRE-PUB), the pups of treated pregnant females received the same dose of EE2 in the pubertal period. Gerbils in the control group were not exposed to EE. In all the groups, the gerbils ($n = 4$) were killed at 12 months (360 days) of age.

Organ senescence is associated with several diseases, including cancer, diabetes, obesity, and cardiovascular disease. In the case of ovaries, aging is characterized by gradual declines in ovarian follicle number and function, which leads to infertility. The aging process is affected by numerous factors, including lifestyle and drugs, as well as environmental and genetic factors.⁹ Thus, it is important to understand how EDCs, like EE2, may alter ovarian development and the consequences of these changes during aging.

Mongolian gerbils (*Meriones unguiculatus*) are rodents used as experimental models to study changes in aging, including in reproductive organs, such as the ovary.¹⁰ In these animals, exposure to low doses of EE2 during prenatal or pubertal periods promotes morphological and functional changes in the senile male and female prostate.^{11,12} Other EDCs, such as quinnestrol and levonorgestrel, promote changes in hormonal levels and receptor and protein expression in those animals.^{13,14}

However, the effects of exposure to EE2, during prenatal and pubertal periods, in reproductive function and ovary morphology of old gerbils remain unclear. On these bases, this study was developed to evaluate the effects of exposure to low doses of 17 α -ethinylestradiol in prenatal and/or pubertal periods in ovary morphology, follicle availability, and hormonal levels in 12-month-old female gerbils.

Materials and methods

Animals and experimental design

The female gerbils (*Meriones unguiculatus*) used in this study were maintained in a vivarium in São Paulo State University (UNESP) (São José do Rio Preto) inside polyethylene cages under

controlled conditions of temperature (23°C) and light (12 hours dark/12 hours light). They were provided filtered water in glass bottles, and rodent food *ad libitum* composed by 23% of protein, 12% of minerals, 5% of fiber, and 4% of total lipids (Nestle Purina PetCare, St. Louis, MO). Animal handling and experiments were in accordance with ethical principles of animal research and were approved by the Committee for Animal Research (protocol n° 020/09 CEEA) of UNESP.

Four nulliparous adult female gerbils (90–120 days) were maintained with a male of the same age for the formation of different families and separated into four different groups. Mating was confirmed by the presence of spermatozooids in the vaginal smear (the day 0 of pregnancy).

As shown in Fig. 1, in the EE/PRE group, four pregnant females received 15 µg/kg/day of 17 α -ethinylestradiol (Sigma, St. Louis, MO, USA) diluted in the total volume of 100 µl Nujol® mineral oil (CAS 8020-83-5; Sigma-Aldrich) by gavage. The dose of EE2 used in the experiment is similar to the dose found in the contraceptive pills.¹⁵ Pregnant females in the EE/PRE groups received EE2 from the 18th to the 22nd day, taking into account that the gestational time of the gerbil is 26 days. The treatment was performed in a window of susceptibility of follicle development, considering that primordial follicle formation occurs during late fetal and early postnatal periods in rodents.⁷ Then, four pups of the females treated during the gestational period formed the EE/PRE group.

In the EE/PUB group, 42-days-old female gerbils received by gavage the same dose of EE2 for 1 week during the puberty period.^{16,17} The EE/PRE-PUB group was formed by four pups of pregnant female treated with EE2 during the gestation and received the same dose of EE2 during the pubertal period. In the control

group, the females did not receive any treatment. During the experimental procedure, we did not utilize vehicle controls because previous studies by our group had shown no significance between the vehicle group and intact animals.^{18–20}

The 12-month-old animals were euthanized by anesthesia in CO₂ followed by decapitation. After death, the females were weighed and ovaries were removed and also weighed. Before euthanasia, all females were cycled, and at the time of euthanasia all were in the proestrus phase of the estrous cycle, as described in previous studies.²¹

The normal estrous cycle of Mongolian gerbils was 4–6 days long. However, several irregularities could be found in the late phases of the cycle, such as pseudopregnancy. The proestrus phase, the beginning of the estrous cycle, has a more constant duration and few variations in serum levels of estrogen and progesterone when compared with the other phases.^{21,22} Thus, the cycling of animals in this phase facilitates the standardization of the evaluation of tissues highly dependent on the levels of sex hormones, such as the ovary.

Serum hormone levels

The blood of 12-month-old female gerbils from experimental groups was collected at decapitation. The blood was centrifuged (3000 rpm for 20 min) and stored at –80°C. The serum hormonal levels were determined in duplicate by Enzyme-Linked Immunosorbent Assay (ELISA). In this study, high sensitivity kits (Testosterone ELISA kit No. 582701 and 17 β -estradiol ELISA kit No. 501890, Cayman Chemical Company, MI, USA) following the instructions of manufacturers were used. The sensitivity was 20 pg/ml for estradiol and 6 pg/ml for testosterone. The limit of detection for estradiol was 6 pg/ml. The readings were performed using SpectraMax Plus 384, at 405 nm (Molecular Devices, Sunnyvale, CA, USA).

Morphological and morphometric analysis

Both ovaries of the experimental groups were fixed in methacarn in a 1:3:6 proportion of acetic acid, chloroform, and methanol and subjected to paraffin (Histosec, Merk) inclusion. Both gonads of each animal ($n = 4$) were sectioned (5 μ m) using a semiautomatic rotating microtome (RM2245, Leica Wetzlar, Germany) and were subjected to Hematoxylin-Eosin (HE),²³ Periodic Acid Schiff (PAS),²⁴ and Gomori's trichrome (GT)²⁵ for morphological, morphometrical, and histopathological analysis.

The images for this analysis were scanned using ScanScope[®] AT Turbo (Leica Wetzlar, Germany) and analyzed using Aperio ImageScope[®] Software version 12.4.05.043 (Leica Wetzlar, Germany). To aid in histological analysis, an optical microscope (Leica Byosystems DM750 Wetzlar, Germany) was also used. The morphometric analysis of the thickness of epithelium and the tunica albuginea was performed using Software Image-Pro Plus version 6.0 for Windows (Media Cybernetics, Inc., Silver Spring, MA).

The number of ovarian follicles was quantified, in HE staining, according to maturation stage, and classified as primordial, initial primary, late primary, secondary, and mature.^{26,27} Only follicles with evident oocytes were considered upon visualization of the nucleus or cytoplasmic delimitation.

The number of atretic follicles in PAS staining was also performed. The atretic follicle was identified by overall shrinkage, oocyte degeneration, and thickening of the basement membrane.^{28,29}

Atretic follicles were easily identified in PAS staining considering the thickening of the basement membrane.

The quantification of the number of corpus luteum, in HE staining, was also performed.²⁶ The quantification of the area occupied by lutein cells in ovarian tissue was also analyzed by stereological multipurpose graticulate with 130 points and 10 test lines.³⁰ Quantification was performed in 400 \times magnification, with all ovarian tissue evaluated in the process. Both ovaries of all the animals ($n = 4$) were included in the counting and analyzed in duplicate.

The interstitial gland is a poorly characterized structure in ovarian morphology, presenting cells with clear cytoplasm and intracytoplasmic granules in HE staining.³¹ The morphological analysis of the interstitial gland included the comparison between HE, PAS, and GT stains to better clarify the components of the structure. The morphometrical analysis was done using the Weibel's method. As in the morphometry of the interstitial gland, lipofuscin was quantified by Weibel's method after the morphological identification in HE of cells with lipofuscin granules.³²

The thickness of the ovarian epithelium was measured between the interface of the apical region of the epithelium and the basal membrane. The thickness of the tunica albuginea was measured between the end of the ovarian epithelium and the end of the dense irregular connective tissue. The analysis was carried out using methods previously described,¹² being used 10 microscopic 400 \times magnification fields per animal and 10 measurements per field, with a total of 400 measurements per experimental group. The measurement fields of the ovarian epithelium and the tunica albuginea were randomly selected. Different representative spots were considered, excluding areas with cystic degeneration³³ or histological artifacts.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software version 5.00 (Inc., La Jolla, CA, USA). The morphological and morphometric data obtained in this study were subjected to parametrical tests, including analysis of variance (ANOVA), followed by multiple comparison between groups by the Tukey test. The hormonal data were subjected to nonparametric Kruskal-Wallis and Dunn comparative tests. The level of significance adopted was $P < 0.05$, expressed as mean and standard deviation.

Results

Exposure to EE2 did not change biometric parameters

The body and ovary weight and relative weight of the ovary are presented in Table 1. EE/PUB and EE/PRE groups present heavier ovaries than the control and EE/PRE-PUB groups; however, no significant statistical difference was found between the biometric data in experimental groups.

Higher levels of serum hormones were found in treated groups, especially in EE/PUB

The serum levels of estradiol and testosterone are shown in Table 2. The 12-month-old females of the EE/PRE and EE/PUB groups showed a significant increase in estradiol levels when compared to the control group. As for testosterone levels, only females in EE/PUB group showed a significant increase when compared to

Table 1. Biometric data of 12-month-old female gerbils from experimental groups

Parameters	Control group	EE/PRE group	EE/PUB group	EE/PRE-PUB group
Body weight (g)	66.86 ± 6.86	72.20 ± 6.89	68.92 ± 12.26	61.20 ± 8.32
Ovary weight (mg)	30.00 ± 10.00	70.00 ± 10.00	90.00 ± 100.00	40.00 ± 30.00
Relative weight of the ovaries ($\times 10^{-3}$)	0.51 ± 0.21	1.04 ± 0.15	1.36 ± 1.70	0.72 ± 0.42

The data are expressed as mean ± standard deviation ($n = 4$).

Table 2. Serological data of 12-month-old female gerbils from experimental groups

Parameters	Control group	EE/PRE group	EE/PUB group	EE/PRE-PUB group
Estradiol (pg/ml)	7.95 ± 2.20a	21.23 ± 3.56b*	19.90 ± 5.70b*	13.40 ± 1.49ab
Testosterone (pg/ml)	1001.00 ± 1001.00a	1450.00 ± 250.00ab	3240.00 ± 190.00b**	1460.00 ± 560.00ab

The data are expressed as mean ± standard deviation. $n = 4$.

Significant difference between the groups was highlighted by superscript letters (a,b).

* $P < 0.05$, ** $P < 0.01$.

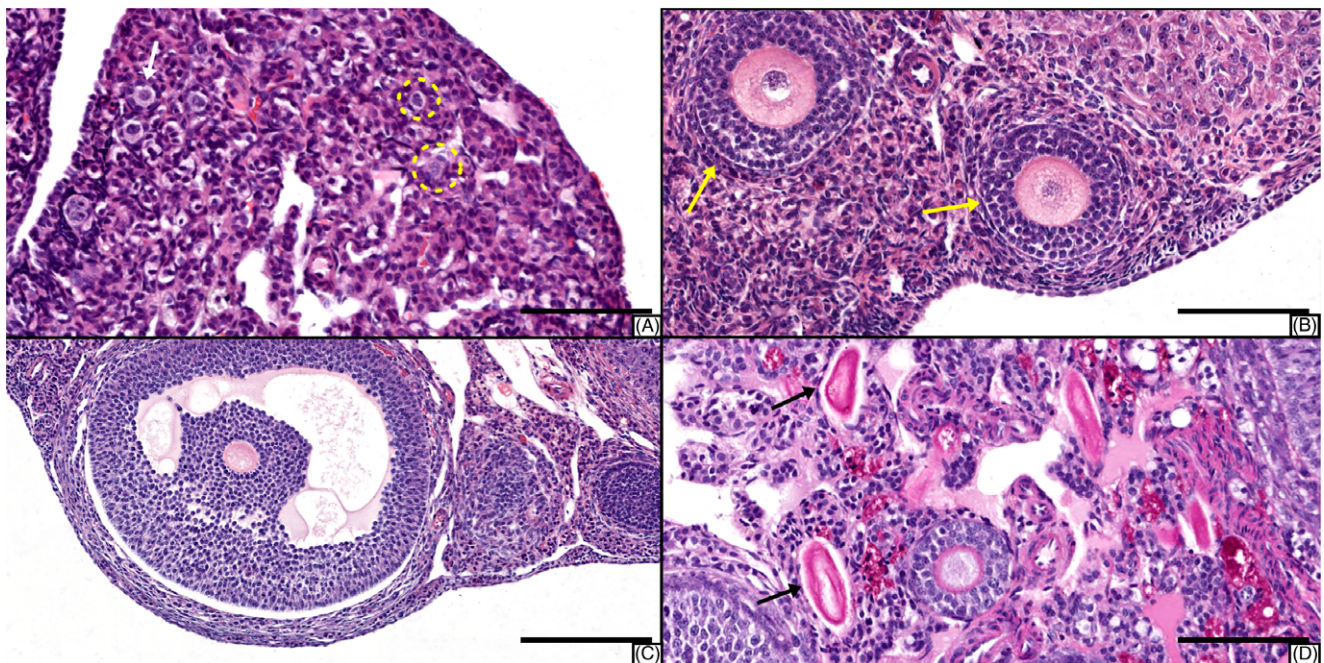


Fig. 2. Histological sections of follicles stained with Hematoxylin and Eosin (HE) (A, B, C) and Period Acid Schiff (PAS) (D). The images highlight the morphology of the primordial and primary unilaminar follicles (A), primary multilaminar follicles (B), secondary follicle (C), and atretic follicles (D). Yellow circles: Primordial follicles. White arrow: Primary unilaminar follicle. Black arrows: Atretic follicles. Scale bar of A, B, and D: 50 μ m. Scale bar of C: 100 μ m.

the control group. No significant difference was found when comparing the treated groups with each other.

Exposure to EE2 alters folliculogenesis, especially during pubertal exposure

No evident morphological changes were observed in the follicles of the experimental groups. The ovarian tissue of most animals showed a low density of viable follicles, with the presence of ovarian stroma being predominant. The morphology of viable and atretic follicles was shown in Fig. 2.

The data of the follicle count can be seen in Table 3. A significant increase in primordial follicle count was found in the EE/PUB and EE/PRE-PUB groups when compared to the control and

EE/PRE groups. There was no statistical difference between primordial follicle count in EE/PUB and EE/PRE-PUB groups.

Considering the count of primary unilaminar follicles, a significant reduction was showed in EE/PRE group when compared to EE/PRE-PUB, control, and EE/PUB. In the analysis of primary multilaminar follicles, all treated groups showed a significant reduction in count when compared to the control group. The greatest reduction was observed in the EE/PRE group. There was no statistical difference between the treated groups.

As for secondary follicles, most animals did not have a large amount of this group of follicles. A significant reduction of secondary follicle count was showed in EE/PUB when compared to control, EE-PRE and EE/PRE-PUB groups. Finally, only the EE/PUB and EE/PRE-PUB groups had mature follicles in some of the

Table 3. Morphometric data of 12-month-old female gerbils from experimental groups

Parameters	Control group	EE/PRE group	EE/PUB group	EE/PRE-PUB group
Number of primordial follicles	2.54 ± 2.69a	3.20 ± 2.23a	4.91 ± 2.94b*	7.20 ± 5.70b*
Number of primary unilaminar follicles	3.74 ± 3.24a	1.89 ± 1.69b*	2.61 ± 2.21a	4.54 ± 2.79a
Number of primary multilaminar follicles	2.74 ± 1.58a	0.89 ± 0.80b*	1.67 ± 1.71b*	1.72 ± 1.67b*
Number of secondary follicles	0.31 ± 0.58a	0.17 ± 0.45a	0.03 ± 0.17b*	0.47 ± 0.08a
Number of mature follicles	0.00 ± 0.00	0.00 ± 0.00	0.16 ± 0.57	0.05 ± 0.22
Number of corpus luteum	1.13 ± 0.91a	2.35 ± 0.86b***	0.83 ± 0.86a	0.88 ± 1.05a
Morphometry of corpus luteum (%)	26.81 ± 34.66a	38.60 ± 44.03b***	10.16 ± 24.64c***	16.30 ± 33.71ac
Number of atretic follicles	19.60 ± 12.19	21.14 ± 7.60	19.67 ± 8.29	16.71 ± 15.69
Morphometry of interstitial gland (%)	6.82 ± 11.19a	8.15 ± 14.07a	23.04 ± 15.45b***	16.74 ± 17.31c***
Morphometry of lipofuscin (%)	2.27 ± 3.29a	1.62 ± 3.42a	3.64 ± 3.91b***	2.71 ± 3.82b**
Ovarian epithelium thickness (µm)	2.17 ± 0.79a	2.49 ± 0.80a	2.41 ± 0.93a	2.86 ± 0.98b***
Tunica albuginea thickness (µm)	8.12 ± 3.84a	11.23 ± 6.63b***	15.75 ± 17.25c***	19.01 ± 13.50d***

The data are expressed as mean ± standard deviation. $n = 4$.

Significant difference between the groups was highlighted by superscript letters (a,b,c,d). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

animals. There was no statistically significant difference between groups.

Exposure to EE2 in the prenatal period increases the number of corpus luteum in 12-month-old gerbils

There were no changes in the morphology of the cells that make up the corpus luteum. However, as seen in Table 3, 12-month-old females exposed to EE2 during the prenatal period had a greater amount of corpus luteum compared to the control, EE/PUB group, and EE/PRE-PUB group. EE/PRE was the group with the largest number of corpus luteum, followed by control, EE/PRE-PUB, and EE/PUB. In addition, considering the morphometry of this structure, the EE/PRE group also had the largest relative proportion lutein cells in the ovary, with a significant increase when compared to other experimental groups. Finally, the proportion of lutein cells in ovaries in the EE/PUB group was significantly reduced when compared to the control group and also to the EE/PRE group.

Exposure to EE2 did not change the number of atretic follicles

Table 3 shows the data related to quantification of atretic follicles. The atretic follicles were easily identified by strong reactivity to PAS as shown in Fig. 2. No significant changes were observed in the morphology and quantification of atretic follicles in the treated groups when compared to the control group.

Exposure to EE2 during puberty increases the frequency of interstitial gland cells in ovary of 12-month-old gerbils

The cells of the interstitial gland presented a clear cytoplasm with eosinophilic granules in HE staining (Fig. 3a, 3d, 3g, 3j). Poor reactivity was observed in PAS staining (Fig. 3b, 3e, 3h, 3k), with a predominance of the reactivity in cytoplasmic granules and cell membrane. Some of the cells have more than one evident nucleus.

Lipid droplets were also evident in interstitial gland cells and could be identified by circular empty spaces (Fig. 3).

In all groups, the cells of the interstitial gland were arranged in strings (Fig. 3a–3c) or nests (Fig. 3g–3i). In the control and EE/PRE groups, the cells of the interstitial gland were located predominantly below the ovarian epithelium (Fig. 3a–3f). On the other hand, in the EE/PUB and EE/PRE-PUB groups, a higher number of interstitial gland cells were noticed, occupying more extensive areas in the ovarian cortex. In these groups, in addition to the increasing number of cells, hypertrophy of interstitial cells and more evident cytoplasmic granules were also observed (Fig. 3g–3i). The glandular formations are surrounded by fibrous tissue and permeated by septa of connective tissue, which are better evidenced in the GT staining.

The morphometry data of the interstitial gland are shown in Table 3. All treated groups had a higher percentage of interstitial cells, with statistical difference in the EE/PUB and EE/PRE-PUB groups when compared to the control group. The highest percentage was observed in the EE/PUB group, followed by EE/PRE-PUB, EE/PRE, and control groups. The groups treated during puberty also showed a statistical difference when compared to the EE/PRE group, and the highest percentage in the EE/PUB group was also significant when compared to the EE/PRE-PUB group.

Exposure to EE2 during puberty increases the deposits of lipofuscin in ovaries of 12-month-old gerbils

All experimental groups showed deposits of lipofuscin along the ovarian tissue. The pigment was identified by HE staining through deposits of brownish material in the cytoplasm (Fig. 3a, 3d, 3g, 3j). Lipofuscin also showed PAS reactivity with variable intensity along the section (Fig. 3b, 3e, 3h, 3k). In GT staining, the identification of the deposited material is difficult, but it can be seen in shades that varied from dark green to brown (Fig. 3c, 3f, 3i, 3l). The deposits of this pigment occurred mainly in interstitial gland cells.

As seen in Table 3, a greater area occupied by cells with lipofuscin deposit was observed in the EE/PUB and EE/PRE-PUB

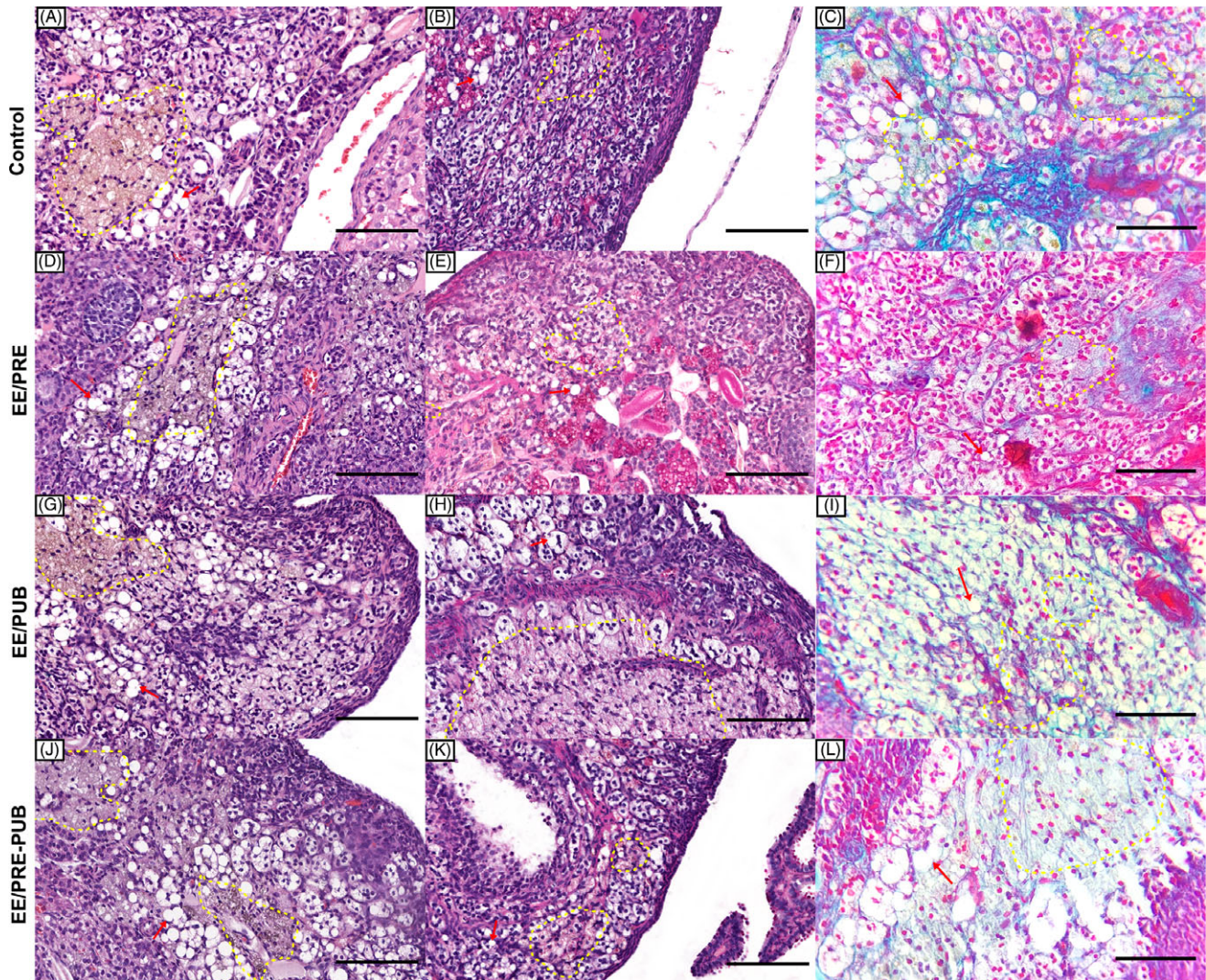


Fig. 3. Histological sections of interstitial gland and lipofuscin stained with Hematoxylin and Eosin (HE) (A, D, G, J), Period Acid Schiff (PAS) (B, E, H, K), and Gomori's Trichrome (GT) (C, F, I, L). Lines 1, 2, 3 and 4 represent, respectively, the Control, EE/PRE, EE/PUB, and EE/PRE-PUB groups. The histological sections highlight the interstitial gland composed by cells with clear cytoplasm with intracytoplasmic granules and their arrangements in strings (more evident in Control group) or nests (more evident in EE/PUB group), permeated by fibrous septa. Lipid droplets (red arrows) can be seen in interstitial gland cells. Lipofuscin (yellow dashed) can be identified by brownish material in HE stain, PAS reactivity and dark green to brown in GT stain. Scale bar: 50 μ m.

groups when compared to the control and EE/PRE groups. There was no difference between the EE/PRE group and the control group or between the EE/PUB group and the EE/PRE-PUB group.

Exposure to EE2 in EE/PRE-PUB group increases the thickness of ovarian epithelium of 12-month-old gerbils

As seen in Fig. 4, the ovarian epithelium of 12-month-old female gerbils often varied from simple squamous epithelium (Fig. 4Aa–4d) to simple cuboidal (Fig. 4e, 4f). In the case of the EE/PRE-PUB group, areas of simple columnar epithelium were also observed (Fig. 4g, 4h).

The data regarding the effect of exposure to 17 α -ethinylestradiol on the ovarian epithelium are shown in Table 3. The higher epithelial thickness was found in the EE/PRE-PUB group, followed by the EE/PRE, EE/PUB, and control groups. The ovaries of females in the EE/PRE-PUB group showed a statistically significant increase

in epithelial thickness when compared to the control, EE/PRE, and EE/PUB groups. There was no difference between EE/PRE, EE/PUB, and control groups.

Exposure to EE2 increases the thickness of tunica albuginea of the ovary of 12-month-old gerbils

In all experimental groups, the tunica albuginea was formed by dense irregular connective tissue, which showed individual non-systematic variation in cellularity and deposit of collagen fibers. The Gomori's trichrome stain highlights the collagen composition of tunica albuginea with a green color (Fig. 4b, 4d, 4f, 4h).

In the statistical analysis, there was a significant increase in the thickness of tunica albuginea between the ovaries of all females in the experimental groups. As seen in Table 3, the thickest tunica albuginea was found in the EE/PRE-PUB group, followed by the EE/PUB, EE/PRE, and control groups.

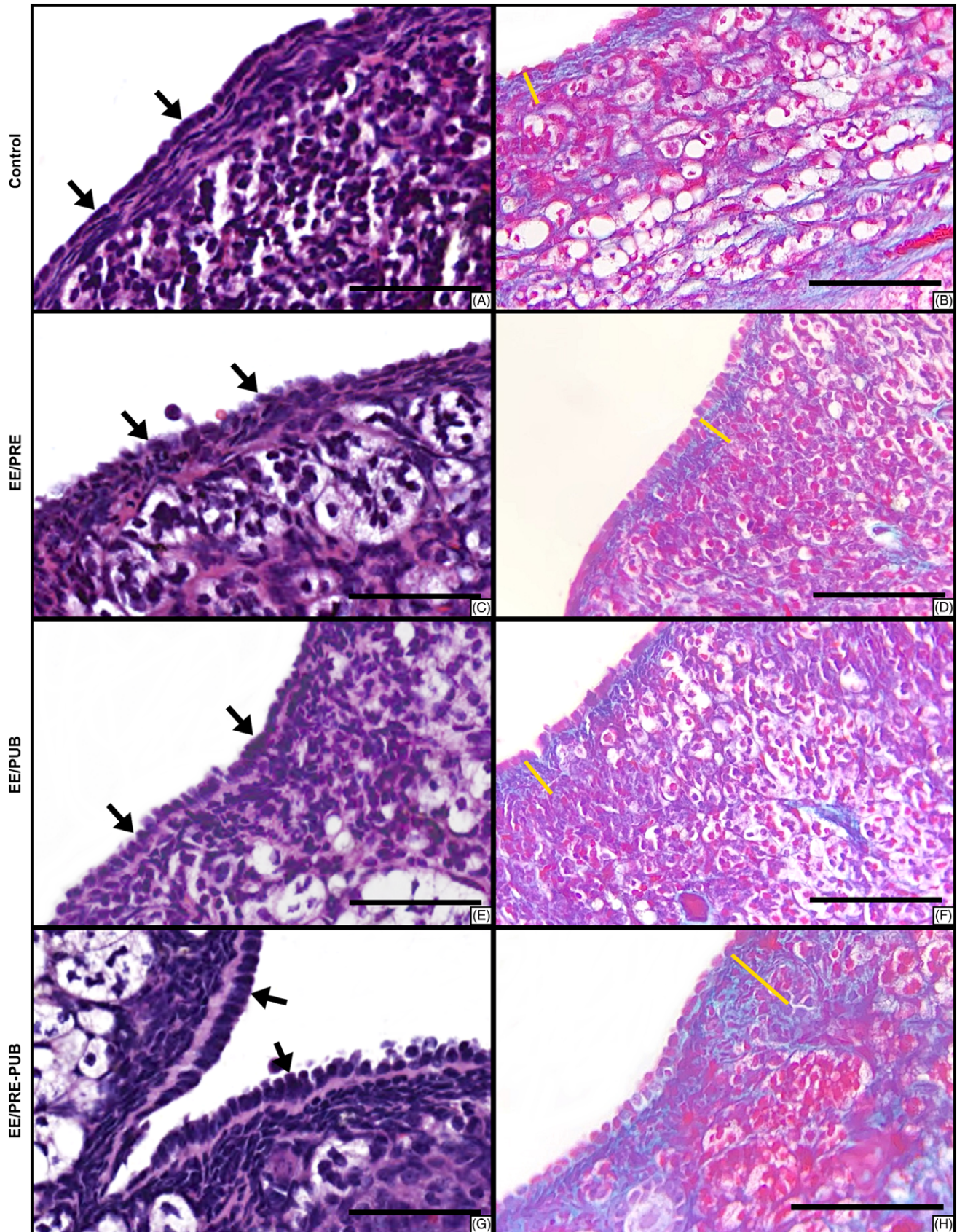


Fig. 4. Histological sections of ovarian superficial epithelium and tunica albuginea stained with Hematoxylin and Eosin (HE) (A, C, E, G) and Gomori's Trichrome (GT) (B, D, F, H). Ovarian epithelium (black arrows) varied between simple squamous epithelium (Control and EE/PRE groups), simple cuboidal (EE/PUB group), and simple columnar (EE/PRE-PUB). The thickness of tunica albuginea (yellow line) was significant higher in treated groups. Scale bar: 50 μ m.

Discussion

The senescence period is marked by a substantial reduction in ovarian follicles, especially mature follicles.^{34,35} Considering the importance of folliculogenesis and the maintenance of follicle levels for female fertility, studies have been developed to identify protective and aggressive factors in this process.^{7,8}

Exposure to EDCs in critical periods of development alters the hypothalamic-pituitary-gonadal (HPG) axis and changes gonadal hormonal production.³⁶ We found that exposure to EE2 during puberty increases the levels of testosterone and estradiol. These changes may occur due to an imbalance in regulatory feedback mechanism of HPG axis.^{8,12} Furthermore, exposure during prenatal period also increases the levels of estradiol. It is possible that the exposure during developmental periods promotes epigenetic changes in critical signaling pathways, such as kisspeptin expression.⁸

In addition, previous studies showed that early life exposure to endocrine-disrupting chemicals may lead to morphophysiological and epigenetic changes in later life.^{37–39} Micro-RNA-224 (miRNA-224) is an epigenetic factor that regulates the expression of steroidogenic genes, as CYP19A1, and acts in folliculogenesis. Prenatal exposure to EDCs could elevate the expression of miRNA-224 and lead to ovarian pathological conditions.⁴⁰ Thus, it is suggested that the prenatal and/or pubertal exposure to EE2 can lead to long-term pathologic effects by epigenetic changes. In this case, new studies should be developed to understand the epigenetic changes that lead to folliculogenesis deregulation showed in our study.

This study demonstrated that exposure to low doses of EE2 in prenatal and/or pubertal periods promoted changes in folliculogenesis in mature gerbils. The treated groups showed a higher primordial follicle count, especially in mature gerbils that received treatment during puberty. On the other hand, the pre-antral follicle (primary and secondary) count was reduced in these groups.

Ovarian folliculogenesis is influenced by an interaction between granulosa cells, theca cells, and hormones.⁴¹ The role of estrogen in folliculogenesis is still under study, but it is known that 17 β -estradiol stimulates follicular growth and mitosis of granulosa cells, in addition to playing a role in the maturation of antral follicles and in ovulation.^{42–44} On the other hand, it has been shown that exposure to high levels of ethinylestradiol can alter the follicle morphology, with formation of follicular cysts and follicles with several oocytes.^{8,45,46}

Primordial follicles are formed in the latter half of the fetal life and in the neonatal period in mice, while folliculogenesis occurs throughout puberty and sexual life. The formation of primordial follicles consists of breaking the germ cell nests and depends on apoptosis for their correct formation.^{9,47,48} In neonatal period, exposure to high levels of estrogen reduces the process of apoptosis, impairing the formation of normal primordial follicles.^{9,49}

In this study, the mature gerbils treated with low doses of EE2 in prenatal period did not present significant changes in the number of primordial follicles, suggesting that changes in folliculogenesis could be more evident in cases of early neonatal exposure or in the administration of high doses of estrogen as reported by Zhang and collaborators.⁷ Thus, it is suggested that exposure to low doses of EE2 in prenatal period did not affect the mechanism of formation of these follicles.

In addition, the quantity of primordial follicles was higher in mature gerbils treated during puberty. These results could reflect the decrease in follicular activation after pubertal exposure.

Some studies have shown that estrogen compounds promote follicle activation^{50,51} and others showed the opposite effect.^{5,52} These different results may be due to species differences and different mechanisms of action of exogenous estrogen compounds. In this study, it was suggested that exposure to low doses of EE2 during puberty could reduce follicle activation with higher count of primordial follicles in mature gerbils.

Folliculogenesis can be divided into pre-antral – culminating in the formation of the secondary follicle – and antral, forming the mature follicle. Estradiol plays an important role in antral folliculogenesis and ovulation; however, its role in preantral folliculogenesis is still not defined.⁵³ Previous studies showed that exposure to low doses of EE in the prenatal period promoted an increase in the number of primary follicles in other periods of development, suggesting that the compound promotes changes in folliculogenesis.⁵⁴

In this study, the reduction of preantral follicles in the mature animals exposed to EE2 in prenatal period and at puberty suggested that low doses of EE2 may promote changes in preantral folliculogenesis. Thus, it is suggested that the exposure to contraceptives in developmental periods, such as prenatal and puberty, may affect ovarian function in old age.

In addition to changes in folliculogenesis, exposure to low doses of ethinylestradiol in the prenatal period promoted an increase in the quantification and morphometry of the corpus luteum in mature gerbils, while higher serological levels of estradiol are also being observed in these animals. On the other hand, exposure to EE2 during the puberty period determined a reduction in the corpus luteum morphometry in mature gerbils, also accompanied by higher serological levels of estradiol.

Previous studies have already demonstrated the influence of ethinylestradiol on the stages of estrous cycle of female rodents, with delay or late onset being observed in females exposed to certain concentrations of exogenous estradiol analogs.⁵⁵

Among the endocrine mechanisms involved in the development and maintenance of the corpus luteum, the elevated plasma estrogen concentration from the preovulatory follicle stands out as a preponderant factor, as it determines a preovulatory peak of LH and, consequently, ovulation and luteinization. Likewise, estradiol is also related to the degeneration of the structure of the corpus luteum, since they are related to the secretion of PGF2-a, a substance responsible for, among other effects, decreasing blood flow to the corpus luteum and degeneration of its capillaries.^{13,55,56}

In gerbils, females that have not copulated present a rapid involution of the corpus luteum within 3 to 6 days after their formation. However, remaining luteal cells may be present for longer periods of time.^{57,58} Considering the short estrous cycle of gerbils, the cross-sectional analysis of the corpus luteum may reflect the presence of newly formed corpus luteum and corpus luteum remaining from previous cycles.

Thus, it is possible to assume that exposure to EE2 during the prenatal period may contribute to a way of favoring the luteinization phase and the formation of the corpus luteum in mature animals. It is also possible that this exposure disrupts the normal estrous cycle of gerbils, as demonstrated in other rodents,⁵⁵ favoring the slower evolution of the corpus luteum. In addition, there was a proliferation of other ovarian structures in 12-month-old gerbils exposed to EE2 during puberty, such as the interstitial gland, which could justify the significant reduction in the morphometry of the corpus luteum in this group, not accompanied by a significant reduction in the quantification of these structures.

In addition to the above, this study did not reveal changes in follicular atresia in 12-month-old female gerbils exposed to EE2 during the prenatal period and/or puberty. Previous studies have shown that exposure to exogenous estrogens promoted an increase in follicular atresia in a dose-dependent manner.^{59–61} Thus, based on the results of this study, it is suggested that low doses of EE2 are not sufficient to promote significant increases in follicular atresia.

The interstitial glands, described in the human ovary in 1902,⁶² are originated from cells of the theca interna of atretic follicles.⁶³ Initially, the primary interstitial gland is located below the epithelial surface, in the primary portion of the ovarian cortex, even before puberty. During old age, the gland increases in size, and new areas are formed along the ovarian stroma, characterizing the secondary interstitial gland.³¹

The cells of the interstitial gland have an important steroidogenic function, being capable of producing high androgen levels.^{64,65} The postmenopausal ovary is hormonally active, being viewed as the expression of steroidogenic enzymes and the production of steroids such as testosterone and estradiol.^{66–69} The secondary interstitial gland, formed during old age, has an even more evident steroidogenic function, being essential for the maintenance of ovarian function in the post-menopausal period.³¹

The morphological analysis conducted in this study suggests that the interstitial gland had lipidic and glycoproteic components, considering the findings in HE and PAS stains. In this study, all groups had a secondary interstitial gland, although this was more evident in the EE/PUB and EE/PRE-PUB groups.

Treatment with low doses of EE during puberty increased the number of the interstitial gland cells in 12-month-old gerbils. This increase was even more pronounced in the EE/PUB group, in which higher levels of testosterone and estradiol were detected, showing the importance of the gland in steroidogenesis. These results suggest that the use of contraceptives during puberty can contribute to the maintenance of ovarian steroidogenic function in old age. However, it is important to note that prolonged exposure to estradiol increases the risk of some diseases, including breast and endometrial cancer.^{70,71} On the other hand, estrogen has a cardioprotective function,⁷² and the maintenance of steroidogenic function during senescence may help to reduce the risk of cardiovascular diseases.

However, the intense activity of the interstitial gland can lead to the accumulation of reactive oxygen species, contributing to the change in folliculogenesis and impairing the viability of ovarian follicles.⁷³

Lipofuscin is a pigment formed by deposits of oxidized protein and lipid,⁷⁴ visualized in HE staining as brownish-yellow deposits. Popularly known as the “aging pigment,” lipofuscin is formed from the oxidation of cellular components.⁷⁵

In this study, exposure to EE during puberty increased the deposits of lipofuscin in ovarian tissue of mature animals. Pigmentation was present mainly in the cells of the interstitial gland. The pigment was easily identified by brownish-yellow deposits in HE and PAS reactivity.

Previous studies showed that the highest deposits of lipofuscin in ovarian tissue occurred in nulliparous mice.⁷⁶ Nulliparity is associated with higher levels of estradiol when compared to multiparity.⁷⁷ In our study, mature gerbils exposed to EE2 during puberty, especially in EE/PUB group, had higher serological levels of estrogen and also higher deposition of lipofuscin.

Lipofuscin has chemical properties that can culminate in disorders of cellular metabolism. The pigment is also associated with deposits in macrophages, imbalance of folliculogenesis and ovarian

function, increased oxidative stress and DNA damage.^{76,78} Thus, it is suggested that the greater deposits of lipofuscin, associated with exposure to EE, may be a predisposing factor to ovarian dysfunction and inflammatory and neoplastic pathologies in the ovarian tissue. Other studies should be developed to investigate the association of exposure to EE2 and the pathologic conditions.

This study showed that longest exposure to low doses of EE2, in the case of the EE/PRE-PUB group, led to a significant increase in the height of the ovarian superficial epithelium in 12-month-old gerbils. Previous studies have shown that high levels of estrogen promote the proliferation of ovarian epithelium and increase the risk of ovarian epithelial cancer.⁷⁹ The interaction between estrogen and its receptors promotes genetic instability due to frequent replication and recombination of DNA⁸⁰; the stimulation of mitotic pathways⁸¹; and the release of transcription factors that culminate in epithelial proliferation, angiogenesis, and remodeling of the tissue matrix.^{82,83} Previous studies showed that the administration of high doses of estrogen promoted the excessive proliferation of ovarian epithelium of 12-month-old female rats.⁸⁴ Our study reinforces that low doses of ethinylestradiol may lead to thickening of ovarian epithelium. Thus, other studies should be developed to evaluate the effects of low doses of estrogen during prenatal and puberty in the risk of ovarian cancer and other proliferative disorders.

Another important change observed in this study was the thicker tunica albuginea in all experimental groups. The thickening of tunica albuginea is expected during the proestrus,⁸⁵ in aging⁸⁵ and in pathological conditions such as polycystic ovary syndrome.⁸⁶ Furthermore, it has been shown that prolonged exposure to exogenous androgens promotes thickening of the tunica albuginea.⁸⁷

However, the effects of exposure to estrogenic compounds on stromal proliferation are still unclear. Previous studies have shown the synergistic role of estrogen with paracrine factors derived from oocytes, especially from the transforming growth factor-beta (TGF- β) family, in folliculogenesis.⁸⁸ Thus, it is suggested that the proliferation of the tunica albuginea is secondary to this interaction, since these factors also stimulate fibrogenesis and proliferation of fibroblasts. However, new studies must be developed to better clarify the alteration found.

Considering the widespread use of synthetic estrogens in contraceptive pills, including during initial gestational periods when the mother is still unaware of the pregnancy, and the greater environmental exposure to this compound, adequate clinical management of exposure to endocrine disruptors is essential in order to avoid pathological conditions.

Conclusions

The present study reinforces the importance of careful assessment of exogenous estrogen exposure in ovarian morphology and function. Exposure to low doses of 17 α -ethinylestradiol during prenatal and pubertal periods promoted changes in folliculogenesis, luteinization, and endogenous levels of estradiol and testosterone, hyperplasia of the interstitial gland, higher deposits of lipofuscin, increased height of superficial ovarian epithelium, and increased thickness of the tunica albuginea in mature gerbils.

Our results reinforce the need to study exposure to ethinyl estradiol during different stages of development in the context of pathological changes in ovarian morphology and function. Other researches still need to be developed to identify molecular pathways that promote the morphological changes found in the

ovary of mature gerbils exposed to ethinyl estradiol during the pre-natal and/or pubertal periods.

Acknowledgment. The authors acknowledge the Program of Initiation to Scientific, Technological Research and Innovation at UFG (PIVIC/2018-2019).

Financial support. This research received no specific grant from any funding agency, commercial, or not-for-profit sectors.

Conflict of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the Law nº 11.974/2008 that regulates care and use of laboratory animals and has been approved by the Committee for Animal Research (protocol nº 020/09 CEEA) of UNESP.

References

- Fowler PA, Bellingham M, Sinclair KD, *et al.* Impact of endocrine-disrupting compounds (EDCs) on female reproductive health. *Mol Cell Endocrinol.* 2012; 355(2), 231–239. doi: [10.1016/j.mce.2011.10.021](https://doi.org/10.1016/j.mce.2011.10.021)
- Frye CA, Bo E, Calamandrei G, *et al.* Endocrine disruptors: a review of some sources, effects, and mechanisms of actions on behaviour and neuroendocrine systems. *J Neuroendocrinol.* 2012; 24(1), 144–159. doi: [10.1111/j.1365-2826.2011.02229.x](https://doi.org/10.1111/j.1365-2826.2011.02229.x)
- Nozawa K, Nagaoka K, Zhang H, *et al.* Neonatal exposure to 17 α -ethinyl estradiol affects ovarian gene expression and disrupts reproductive cycles in female rats. *Reprod Toxicol.* 2014; 46, 77–84. doi: [10.1016/j.reprotox.2014.03.001](https://doi.org/10.1016/j.reprotox.2014.03.001)
- Ryan BC, Hotchkiss AK, Crofton KM, Gray LEJ. In utero and lactational exposure to bisphenol A, in contrast to ethinyl estradiol, does not alter sexually dimorphic behavior, puberty, fertility, and anatomy of female LE rats. *Toxicol Sci.* 2010; 114(1), 133–148. doi: [10.1093/toxsci/kfp266](https://doi.org/10.1093/toxsci/kfp266)
- Kezele P, Skinner MK. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. *Endocrinology.* 2003; 144(8), 3329–3337. doi: [10.1210/en.2002-0131](https://doi.org/10.1210/en.2002-0131)
- Drummond AE, Fuller PJ. Ovarian actions of estrogen receptor- β : an update. *Semin Reprod Med.* 2012; 30(1), 32–38. doi: [10.1055/s-0031-1299595](https://doi.org/10.1055/s-0031-1299595)
- Zhang H, Nagaoka K, Usuda K, *et al.* Estrogenic compounds impair primordial follicle formation by inhibiting the expression of Proapoptotic Hrk in neonatal rat ovary. *Biol Reprod.* 2016; 95(4), 78. doi: [10.1095/biolreprod.116.141309](https://doi.org/10.1095/biolreprod.116.141309)
- Zhang H, Taya K, Nagaoka K, Yoshida M, Watanabe G. Neonatal exposure to 17 α -ethinyl estradiol (EE) disrupts follicle development and reproductive hormone profiles in female rats. *Toxicol Lett.* 2017; 276, 92–99. doi: [10.1016/j.toxlet.2017.05.014](https://doi.org/10.1016/j.toxlet.2017.05.014)
- Zhang J, Chen Q, Du D, *et al.* Can ovarian aging be delayed by pharmacological strategies? *Aging (Albany NY).* 2019; 11(2), 817–832. doi: [10.18632/aging.101784](https://doi.org/10.18632/aging.101784)
- Vincent AL, Rodrick GE, Sodeman WA. The Mongolian gerbil in aging research. *Exp Aging Res.* 1980; 6(3), 249–260. doi: [10.1080/03610738008258361](https://doi.org/10.1080/03610738008258361)
- Perez APS, Biancardi MF, Caires CRS, *et al.* Prenatal exposure to ethinylestradiol alters the morphologic patterns and increases the predisposition for prostatic lesions in male and female gerbils during ageing. *Int J Exp Pathol.* 2016; 97(1), 5–17. doi: [10.1111/iep.12153](https://doi.org/10.1111/iep.12153)
- Perez APS, Biancardi MF, Caires CRS, *et al.* Pubertal exposure to ethinylestradiol promotes different effects on the morphology of the prostate of the male and female gerbil during aging. *Environ Toxicol.* 2017; 32(2), 477–489. doi: [10.1002/tox.22252](https://doi.org/10.1002/tox.22252)
- Lv X, Shi D. Combined effects of levonorgestrel and quinestrol on reproductive hormone levels and receptor expression in females of the Mongolian gerbil (*Meriones unguiculatus*). *Zoolog Sci.* 2012; 29(1), 37–42. doi: [10.2108/zsj.29.37](https://doi.org/10.2108/zsj.29.37)
- Su Q-Q, Chen Y, Qin J, Wang T-L, Wang D-H, Liu Q-S. Responses in reproductive organs, steroid hormones and CYP450 enzymes in female Mongolian gerbil (*Meriones unguiculatus*) over time after quinestrol treatment. *Pestic Biochem Physiol.* 2017; 143, 122–126. doi: [10.1016/j.pestbp.2017.08.008](https://doi.org/10.1016/j.pestbp.2017.08.008)
- Thayer KA, Ruhlen RL, Howdeshell KL, *et al.* Altered prostate growth and daily sperm production in male mice exposed prenatally to subclinical doses of 17 α -ethinyl oestradiol. *Hum Reprod.* 2001; 16(5), 988–996. doi: [10.1093/humrep/16.5.988](https://doi.org/10.1093/humrep/16.5.988)
- Pinto-Fochi ME, Negrin AC, Scarano WR, Taboga SR, Góes RM. Sexual maturation of the Mongolian gerbil (*Meriones unguiculatus*): a histological, hormonal and spermatoc evaluation. *Reprod Fertil Dev.* 2016; 28(6), 815–823. doi: [10.1071/RD14074](https://doi.org/10.1071/RD14074)
- Siegford JM, Hadi Mansouri S, Ulibarri C. Normal ontogeny of perineal muscles and testosterone levels in Mongolian gerbils; response to testosterone in developing females. *Anat Rec Part A, Discov Mol Cell Evol Biol.* 2003; 275(1), 997–1008. doi: [10.1002/ar.a.10118](https://doi.org/10.1002/ar.a.10118)
- Santos FCA, Falleiros-Júnior LR, Corradi LS, Vilamaior PSL, Taboga SR. Experimental endocrine therapies promote epithelial cytodifferentiation and ciliogenesis in the gerbil female prostate. *Cell Tissue Res.* 2007; 328(3), 617–624. doi: [10.1007/s00441-007-0381-y](https://doi.org/10.1007/s00441-007-0381-y)
- Scarano WR, de Sousa DE, Campos SGP, Corradi LS, Vilamaior PSL, Taboga SR. Oestrogen supplementation following castration promotes stromal remodelling and histopathological alterations in the Mongolian gerbil ventral prostate. *Int J Exp Pathol.* 2008; 89(1), 25–37. doi: [10.1111/j.1365-2613.2007.00559.x](https://doi.org/10.1111/j.1365-2613.2007.00559.x)
- Scarano WR, Vilamaior PSL, Taboga SR. Tissue evidence of the testosterone role on the abnormal growth and aging effects reversion in the gerbil (*Meriones unguiculatus*) prostate. *Anat Rec Part A, Discov Mol Cell Evol Biol.* 2006; 288(11), 1190–1200. doi: [10.1002/ar.a.20391](https://doi.org/10.1002/ar.a.20391)
- Nishino N, Totsukawa K. Study on the estrous cycle in the Mongolian gerbil (*Meriones unguiculatus*). *Exp Anim.* 1996; 45(3), 283–288. doi: [10.1538/expanim.45.283](https://doi.org/10.1538/expanim.45.283)
- Fochi RA, Perez APS, Bianchi C V, *et al.* Hormonal oscillations during the estrous cycle influence the morphophysiology of the gerbil (*Meriones unguiculatus*) female prostate (skene paraurethral glands). *Biol Reprod.* 2008; 79(6), 1084–1091. doi: [10.1095/biolreprod.108.070540](https://doi.org/10.1095/biolreprod.108.070540)
- Feldman AT, Wolfe D. Tissue processing and hematoxylin and eosin staining. *Methods Mol Biol.* 2014; 1180, 31–43. doi: [10.1007/978-1-4939-1050-2_3](https://doi.org/10.1007/978-1-4939-1050-2_3)
- Kligman AM, Mescon H. The periodic acid-Schiff stain for the demonstration of fungi in animal tissue. *J Bacteriol.* 1950; 60(4), 415–421.
- Gomori G. A rapid one-step trichrome stain. *Am J Clin Pathol.* 1950; 20(7), 661–664. doi: [10.1093/ajcp/20.7_ts.661](https://doi.org/10.1093/ajcp/20.7_ts.661)
- Luo LL, Huang J, Fu YC, Xu JJ, Qian YS. Effects of tea polyphenols on ovarian development in rats. *J Endocrinol Invest.* 2008; 31(12), 1110–1118. doi: [10.1007/BF03345661](https://doi.org/10.1007/BF03345661)
- Takagi K, Yamada T, Miki Y, Umegaki T, Nishimura M, Sasaki J. Histological observation of the development of follicles and follicular atresia in immature rat ovaries. *Acta Med Okayama.* 2007; 61(5), 283–298. doi: [10.18926/AMO/32892](https://doi.org/10.18926/AMO/32892)
- Saidapur SK, Kamath SR. Morphometric study of ovarian follicular growth during the estrous cycle of the laboratory-maintained Indian desert gerbil, *Meriones hurrianae* (Jerdon). *Acta Anat (Basel).* 1993; 148(4), 189–196. doi: [10.1159/000147540](https://doi.org/10.1159/000147540)
- Wang W, Liu H-L, Tian W, *et al.* Morphologic observation and classification criteria of atretic follicles in guinea pigs. *J Zhejiang Univ Sci B.* 2010; 11(5), 307–314. doi: [10.1631/jzus.B0900391](https://doi.org/10.1631/jzus.B0900391)
- Weibel ER. Principles and methods for the morphometric study of the lung and other organs. *Lab Invest.* 1963; 12, 131–155.
- Díaz-Hernández V, Caldelas I, Montañó LM, Merchant-Larios H. Morphological rearrangement of the cortical region, in aging ovaries. *Histol Histopathol.* 2019; 34(7), 775–789. doi: [10.14670/HH-18-078](https://doi.org/10.14670/HH-18-078)
- Angelousi A, Szarek E, Shram V, Kebebew E, Quezado M, Stratakis CA. Lipofuscin accumulation in Cortisol-producing adenomas with and without PRKCA mutations. *Horm Metab Res = Horm und Stoffwechselforsch = Horm Metab.* 2017; 49(10), 786–792. doi: [10.1055/s-0043-116385](https://doi.org/10.1055/s-0043-116385)
- Lunde O, Hoel PS, Sandvik L. Ovarian morphology in patients with polycystic ovaries and in an age-matched reference material. A statistical

- evaluation of 149 cases. *Gynecol Obstet Invest.* 1988; 25(3), 192–201. doi: [10.1159/000293771](https://doi.org/10.1159/000293771)
34. Atkins HM, Willson CJ, Silverstein M, *et al.* Characterization of ovarian aging and reproductive senescence in vervet monkeys (*Chlorocebus aethiops sabaeus*). *Comp Med.* 2014; 64(1), 55–62.
 35. Nichols SM, Bavister BD, Brenner CA, Didier PJ, Harrison RM, Kubisch HM. Ovarian senescence in the rhesus monkey (*Macaca mulatta*). *Hum Reprod.* 2005; 20(1), 79–83. doi: [10.1093/humrep/deh576](https://doi.org/10.1093/humrep/deh576)
 36. Xi W, Lee CKF, Yeung WSB, *et al.* Effect of perinatal and postnatal bisphenol A exposure to the regulatory circuits at the hypothalamus-pituitary-gonadal axis of CD-1 mice. *Reprod Toxicol.* 2011; 31(4), 409–417. doi: [10.1016/j.reprotox.2010.12.002](https://doi.org/10.1016/j.reprotox.2010.12.002)
 37. Brehm E, Rattan S, Gao L, Flaws JA. Prenatal exposure to Di(2-Ethylhexyl) phthalate causes long-term transgenerational effects on female reproduction in mice. *Endocrinology.* 2018; 159(2), 795–809. doi: [10.1210/en.2017-03004](https://doi.org/10.1210/en.2017-03004)
 38. Zha J, Sun L, Zhou Y, Spear PA, Ma M, Wang Z. Assessment of 17 α -ethinylestradiol effects and underlying mechanisms in a continuous, multigeneration exposure of the Chinese rare minnow (*Gobiocypris rarus*). *Toxicol Appl Pharmacol.* 2008; 226(3), 298–308. doi: [10.1016/j.taap.2007.10.006](https://doi.org/10.1016/j.taap.2007.10.006)
 39. Dávila-Esqueda ME, Jiménez-Capdeville ME, Delgado JM, *et al.* Effects of arsenic exposure during the pre- and postnatal development on the puberty of female offspring. *Exp Toxicol Pathol Off J Gesellschaft fur Toxikologische Pathol.* 2012; 64(1–2), 25–30. doi: [10.1016/j.etp.2010.06.001](https://doi.org/10.1016/j.etp.2010.06.001)
 40. Lite C, Ahmed SSSJ, Santosh W, Seetharaman B. Prenatal exposure to bisphenol-A altered miRNA-224 and protein expression of aromatase in ovarian granulosa cells concomitant with elevated serum estradiol levels in F(1) adult offspring. *J Biochem Mol Toxicol.* 2019; 33(6), e22317. doi: [10.1002/jbt.22317](https://doi.org/10.1002/jbt.22317)
 41. Eppig JJ, Pendola FL, Wigglesworth K, Pendola JK. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. *Biol Reprod.* 2005; 73(2), 351–357. doi: [10.1095/biolreprod.105.041798](https://doi.org/10.1095/biolreprod.105.041798)
 42. Bendell JJ, Dorrington J. Estradiol-17 beta stimulates DNA synthesis in rat granulosa cells: action mediated by transforming growth factor-beta. *Endocrinology.* 1991; 128(5), 2663–2665. doi: [10.1210/endo-128-5-2663](https://doi.org/10.1210/endo-128-5-2663)
 43. Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development.* 2000; 127(19), 4277–4291.
 44. Emmen JMA, Couse JF, Elmore SA, Yates MM, Kissling GE, Korach KS. In vitro growth and ovulation of follicles from ovaries of estrogen receptor (ER){alpha} and ER{beta} null mice indicate a role for ER{beta} in follicular maturation. *Endocrinology.* 2005; 146(6), 2817–2826. doi: [10.1210/en.2004-1108](https://doi.org/10.1210/en.2004-1108)
 45. Tarumi W, Itoh MT, Suzuki N. Effects of 5 α -dihydrotestosterone and 17 β -estradiol on the mouse ovarian follicle development and oocyte maturation. *PLoS One.* 2014; 9(6), e99423. doi: [10.1371/journal.pone.0099423](https://doi.org/10.1371/journal.pone.0099423)
 46. Sotomayor-Zárate R, Dorfman M, Paredes A, Lara HE. Neonatal exposure to estradiol valerate programs ovarian sympathetic innervation and follicular development in the adult rat. *Biol Reprod.* 2008; 78(4), 673–680. doi: [10.1095/biolreprod.107.063974](https://doi.org/10.1095/biolreprod.107.063974)
 47. Sawyer HR, Smith P, Heath DA, Juengel JL, Wakefield SJ, McNatty KP. Formation of ovarian follicles during fetal development in sheep. *Biol Reprod.* 2002; 66(4), 1134–1150. doi: [10.1095/biolreprod66.4.1134](https://doi.org/10.1095/biolreprod66.4.1134)
 48. Pepling ME, Spradling AC. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev Biol.* 2001; 234(2), 339–351. doi: [10.1006/dbio.2001.0269](https://doi.org/10.1006/dbio.2001.0269)
 49. Chen Y, Jefferson WN, Newbold RR, Padilla-Banks E, Pepling ME. Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. *Endocrinology.* 2007; 148(8), 3580–3590. doi: [10.1210/en.2007-0088](https://doi.org/10.1210/en.2007-0088)
 50. Rodríguez HA, Santambrosio N, Santamaría CG, Muñoz-de-Toro M, Luque EH. Neonatal exposure to bisphenol A reduces the pool of primordial follicles in the rat ovary. *Reprod Toxicol.* 2010; 30(4), 550–557. doi: [10.1016/j.reprotox.2010.07.008](https://doi.org/10.1016/j.reprotox.2010.07.008)
 51. Wang C, Roy SK. Development of primordial follicles in the hamster: role of estradiol-17beta. *Endocrinology.* 2007; 148(4), 1707–1716. doi: [10.1210/en.2006-1193](https://doi.org/10.1210/en.2006-1193)
 52. Karavan JR, Pepling ME. Effects of estrogenic compounds on neonatal oocyte development. *Reprod Toxicol.* 2012; 34(1), 51–56. doi: [10.1016/j.reprotox.2012.02.005](https://doi.org/10.1016/j.reprotox.2012.02.005)
 53. Edson MA, Nagaraja AK, Matzuk MM. The mammalian ovary from genesis to revelation. *Endocr Rev.* 2009; 30(6), 624–712. doi: [10.1210/er.2009-0012](https://doi.org/10.1210/er.2009-0012)
 54. Patel S, Brehm E, Gao L, Rattan S, Ziv-Gal A, Flaws JA. Bisphenol A exposure, ovarian follicle numbers, and female sex steroid hormone levels: results from a CLARITY-BPA study. *Endocrinology.* 2017; 158(6), 1727–1738. doi: [10.1210/en.2016-1887](https://doi.org/10.1210/en.2016-1887)
 55. Shirota M, Kawashima J, Nakamura T, Kamiie J, Shirota K, Yoshida M. Dose-dependent acceleration in the delayed effects of neonatal oral exposure to low-dose 17 α -ethinylestradiol on reproductive functions in female Sprague-Dawley rats. *J Toxicol Sci.* 2015; 40(6), 727–738. doi: [10.2131/jts.40.727](https://doi.org/10.2131/jts.40.727)
 56. Silvia WJ, Lewis GS, McCracken JA, Thatcher WW, Wilson LJ. Hormonal regulation of uterine secretion of prostaglandin F2 alpha during luteolysis in ruminants. *Biol Reprod.* 1991; 45(5), 655–663. doi: [10.1095/biolreprod45.5.655](https://doi.org/10.1095/biolreprod45.5.655)
 57. Fischer T V, Fisher DL. Effect of gonadotropins on ovulation and ovarian histology in the immature Mongolian gerbil. *Am J Anat.* 1975; 142(3), 391–396. doi: [10.1002/aja.1001420308](https://doi.org/10.1002/aja.1001420308)
 58. Meckley PE, Ginther OJ. Effects of litter and male on corpora lutea of the postpartum Mongolian gerbil. *J Anim Sci.* 1972; 34(2), 297–301. doi: [10.2527/jas1972.342297x](https://doi.org/10.2527/jas1972.342297x)
 59. Kaptaner B, Unal G. Effects of 17 α -ethinylestradiol and nonylphenol on liver and gonadal apoptosis and histopathology in *Chalcalburnus tarichi*. *Environ Toxicol.* 2011; 26(6), 610–622. doi: [10.1002/tox.20585](https://doi.org/10.1002/tox.20585)
 60. Sridevi P, Chaitanya RK, Prathibha Y, Balakrishna SL, Dutta-Gupta A, Senthilkumaran B. Early exposure of 17 α -ethinylestradiol and diethylstilbestrol induces morphological changes and alters ovarian steroidogenic pathway enzyme gene expression in catfish, *Clarias gariepinus*. *Environ Toxicol.* 2015; 30(4), 439–451. doi: [10.1002/tox.21920](https://doi.org/10.1002/tox.21920)
 61. Talsness C, Grote K, Kuriyama S, *et al.* Prenatal exposure to the phytoestrogen daidzein resulted in persistent changes in ovarian surface epithelial cell height, folliculogenesis, and estrus phase length in adult Sprague-Dawley rat offspring. *J Toxicol Environ Health A.* 2015; 78(10), 635–644. doi: [10.1080/15287394.2015.1006711](https://doi.org/10.1080/15287394.2015.1006711)
 62. Limon NM. U de, médecine. F de. Étude histologique et histogénique de la glande interstitielle de l'ovaire. Published online 1901.
 63. Guraya SS, Greenwald GS. A comparative histochemical study of interstitial tissue and follicular atresia in the mammalian ovary. *Anat Rec.* 1964; 149, 411–433. doi: [10.1002/ar.1091490311](https://doi.org/10.1002/ar.1091490311)
 64. Adashi EY. The climacteric ovary as a functional gonadotropin-driven androgen-producing gland. *Fertil Steril.* 1994; 62(1), 20–27. doi: [10.1016/s0015-0282\(16\)56810-1](https://doi.org/10.1016/s0015-0282(16)56810-1)
 65. Erickson GF, Magoffin DA, Dyer CA, Hofeditz C. The ovarian androgen producing cells: a review of structure/function relationships. *Endocr Rev.* 1985; 6(3), 371–399. doi: [10.1210/edrv-6-3-371](https://doi.org/10.1210/edrv-6-3-371)
 66. Brodowski J, Brodowska A, Laszczyńska M, Chlubek D, Starczewski A. Hormone concentrations in the homogenates of ovarian tissue and blood serum in postmenopausal women not using hormone therapy. *Gynecol Endocrinol Off J Int Soc Gynecol Endocrinol.* 2012; 28(5), 396–399. doi: [10.3109/09513590.2012.664189](https://doi.org/10.3109/09513590.2012.664189)
 67. Fogle RH, Stanczyk FZ, Zhang X, Paulson RJ. Ovarian androgen production in postmenopausal women. *J Clin Endocrinol Metab.* 2007; 92(8), 3040–3043. doi: [10.1210/jc.2007-0581](https://doi.org/10.1210/jc.2007-0581)
 68. Havelock JC, Rainey WE, Bradshaw KD, Carr BR. The post-menopausal ovary displays a unique pattern of steroidogenic enzyme expression. *Hum Reprod.* 2006; 21(1), 309–317. doi: [10.1093/humrep/dei373](https://doi.org/10.1093/humrep/dei373)
 69. Laszczyńska M, Brodowska A, Starczewski A, Masiuk M, Brodowski J. Human postmenopausal ovary—hormonally inactive fibrous connective

- tissue or more? *Histol Histopathol.* 2008; 23(2), 219–226. doi: [10.14670/HH-23.219](https://doi.org/10.14670/HH-23.219)
70. Brinton LA, Trabert B, Anderson GL, *et al.* Serum estrogens and estrogen metabolites and endometrial cancer risk among postmenopausal women. *Cancer Epidemiol Biomarkers Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol.* 2016; 25(7), 1081–1089. doi: [10.1158/1055-9965.EPI-16-0225](https://doi.org/10.1158/1055-9965.EPI-16-0225)
 71. Yue W, Wang J-P, Li Y, *et al.* Effects of estrogen on breast cancer development: role of estrogen receptor independent mechanisms. *Int J Cancer.* 2010; 127(8), 1748–1757. doi: [10.1002/ijc.25207](https://doi.org/10.1002/ijc.25207)
 72. Iorga A, Cunningham CM, Moazeni S, Ruffenach G, Umar S, Eghbali M. The protective role of estrogen and estrogen receptors in cardiovascular disease and the controversial use of estrogen therapy. *Biol Sex Differ.* 2017; 8(1), 33. doi: [10.1186/s13293-017-0152-8](https://doi.org/10.1186/s13293-017-0152-8)
 73. Ramalho-Santos J, Amaral S. Mitochondria and mammalian reproduction. *Mol Cell Endocrinol.* 2013; 379(1–2), 74–84. doi: [10.1016/j.mce.2013.06.005](https://doi.org/10.1016/j.mce.2013.06.005)
 74. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell.* 2013; 153(6), 1194–1217. doi: [10.1016/j.cell.2013.05.039](https://doi.org/10.1016/j.cell.2013.05.039)
 75. Jung T, Bader N, Grune T. Lipofuscin: formation, distribution, and metabolic consequences. *Ann N Y Acad Sci.* 2007; 1119, 97–111. doi: [10.1196/annals.1404.008](https://doi.org/10.1196/annals.1404.008)
 76. Urzua U, Chacon C, Espinoza R, Martínez S, Hernandez N. Parity-dependent hemosiderin and lipofuscin accumulation in the reproductively aged mouse ovary. *Anal Cell Pathol (Amst).* 2018; 2018, 1289103. doi: [10.1155/2018/1289103](https://doi.org/10.1155/2018/1289103)
 77. Bernstein L, Pike MC, Ross RK, Judd HL, Brown JB, Henderson BE. Estrogen and sex hormone-binding globulin levels in nulliparous and parous women. *J Natl Cancer Inst.* 1985; 74(4), 741–745.
 78. Wu R, Van der Hoek KH, Ryan NK, Norman RJ, Robker RL. Macrophage contributions to ovarian function. *Hum Reprod Update.* 2004; 10(2), 119–133. doi: [10.1093/humupd/dmh011](https://doi.org/10.1093/humupd/dmh011)
 79. Murdoch WJ, Van Kirk EA. Steroid hormonal regulation of proliferative, p53 tumor suppressor, and apoptotic responses of sheep ovarian surface epithelial cells. *Mol Cell Endocrinol.* 2002; 186(1), 61–67. doi: [10.1016/s0303-7207\(01\)00675-x](https://doi.org/10.1016/s0303-7207(01)00675-x)
 80. Syed V, Ulinski G, Mok SC, Ho S-M. Reproductive hormone-induced, STAT3-mediated interleukin 6 action in normal and malignant human ovarian surface epithelial cells. *J Natl Cancer Inst.* 2002; 94(8), 617–629. doi: [10.1093/jnci/94.8.617](https://doi.org/10.1093/jnci/94.8.617)
 81. Hall JM, Korach KS. Stromal cell-derived factor 1, a novel target of estrogen receptor action, mediates the mitogenic effects of estradiol in ovarian and breast cancer cells. *Mol Endocrinol.* 2003; 17(5), 792–803. doi: [10.1210/me.2002-0438](https://doi.org/10.1210/me.2002-0438)
 82. Moll F, Katsaros D, Lazennec G, *et al.* Estrogen induction and overexpression of fibulin-1C mRNA in ovarian cancer cells. *Oncogene.* 2002; 21(7), 1097–1107. doi: [10.1038/sj.onc.1205171](https://doi.org/10.1038/sj.onc.1205171)
 83. O'Donnell AJM, Macleod KG, Burns DJ, Smyth JF, Langdon SP. Estrogen receptor-alpha mediates gene expression changes and growth response in ovarian cancer cells exposed to estrogen. *Endocr Relat Cancer.* 2005; 12(4), 851–866. doi: [10.1677/erc.1.01039](https://doi.org/10.1677/erc.1.01039)
 84. Perniconi SE, Simões M de J, Simões RDS, Haidar MA, Baracat EC, Soares JMJ. Proliferation of the superficial epithelium of ovaries in senile female rats following oral administration of conjugated equine estrogens. *Clinics (Sao Paulo).* 2008; 63(3), 381–388. doi: [10.1590/s1807-59322008000300016](https://doi.org/10.1590/s1807-59322008000300016)
 85. Joshi CL, Nanda BS, Saigal RP. Histomorphological and histochemical studies on the female genitalia of ageing goat. IV. Histomorphology of ovarian cortex. *Anat Anz.* 1977; 141(1), 19–36.
 86. Bulut G, Kurdoglu Z, Dönmez YB, Kurdoglu M, Erten R. Effects of jnk inhibitor on inflammation and fibrosis in the ovary tissue of a rat model of polycystic ovary syndrome. *Int J Clin Exp Pathol.* 2015; 8(8), 8774–8785.
 87. Amirikia H, Savoy-Moore RT, Sundareson AS, Moghissi KS. The effects of long-term androgen treatment on the ovary. *Fertil Steril.* 1986; 45(2), 202–208. doi: [10.1016/s0015-0282\(16\)49155-7](https://doi.org/10.1016/s0015-0282(16)49155-7)
 88. Emori C, Sugiura K. Role of oocyte-derived paracrine factors in follicular development. *Anim Sci J.* 2014; 85(6), 627–633. doi: [10.1111/asj.12200](https://doi.org/10.1111/asj.12200)