PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIA ANIMAL

GABRIEL RODRIGUES LEAL DE OLIVEIRA

IMPACTO DA EXPOSIÇÃO PERINATAL DE DESREGULADORES ENDÓCRINOS AMBIENTALMENTE RELEVANTES SOBRE A FOLICULOGÊNESE E ORGANIZAÇÃO TECIDUAL OVARIANA DE RATAS ADULTAS

Presidente Prudente - SP
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Dissertação, apresentada ao Programa de Pesquisa e Pós-graduação, Mestrado em Ciência Animal, Universidade do Oeste Paulista, como parte dos requisitos para a sua conclusão.

Orientador:
Prof. Dr. Anthony César de Souza Castilho

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Presidente Prudente, 06 de outubro de 2023.

BANCA EXAMINADORA

_____
Prof. Dr. Orientador. Anthony César de Souza Castilho
Universidade do Oeste Paulista – Unoeste
Presidente Prudente - SP

_____
Prof. Dr. Leonardo de Oliveira Mendes
Universidade do Oeste Paulista - Unoeste
Presidente Prudente - SP

_____
Dra. Ana Caroline Silva Soares
Universidade Estadual Paulista (UNESP) – Instituto de Biociências (IBB)
Botucatu - SP

DEDICATÓRIA

Dedico este trabalho aos meus pais, em especial a minha mãe, que me ensinou o que é o amor.

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Agradeço aos meus pais, que me deram apoio para a finalização do mestrado, mesmo com as intercorrências.

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“A força mais potente do universo é a fé.”

(Madre Teresa de Calcutá.)

RESUMO

Impacto da exposição perinatal de desreguladores endócrinos ambientalmente relevantes sobre a foliculogênese e remodelamento ovariano de ratas adultas

Os desreguladores endócrinos (DEs) são substâncias químicas que se encontram ubíquas no ambiente de seres humanos e animais, com potencial para perturbar o equilíbrio hormonal e interferir nos processos reprodutivos, incluindo a foliculogênese ovariana. No presente estudo, nosso propósito foi investigar os efeitos de uma composição de desreguladores endócrinos (ED-Mix), baseada na exposição ambiental humana, sobre a foliculogênese ovariana e a estrutura do tecido ovariano em ratas adultas cujas mães foram expostas a essa composição durante a gestação e lactação. Para atingir esse objetivo, fêmeas grávidas da linhagem Sprague-Dawley foram distribuídas aleatoriamente em dois grupos experimentais: (i) grupo controle (Control), que recebeu administração de 2ml/kg de óleo de milho por gavagem, e (ii) grupo ED Mix, que recebeu administração de 32,11mg/kg/dia de uma mistura composta por doze compostos (ftalatos, pesticidas, filtros UV, bisfenol A, butilparabeno), diluída em 2ml/kg de óleo de milho, por gavagem. As ratas prenhas e lactantes receberam os tratamentos a partir do 7º dia de gestação (DG7) até o 21º dia pós-natal (DPN21). Após o desmame, no DPN22, houve uma pausa na indução das misturas destes compostos, e as fêmeas da descendência F1 foram sacrificadas no dia 360 pós-natal. Os ovários foram retirados, dissecados e submetidos a análises histológicas. Além disso, amostras de sangue dos animais foram coletadas para avaliação hormonal dos níveis de estradiol e progesterona. As seções de tecido coradas com hematoxilina-eosina e picrosirius foram analisadas quanto a características morfológicas, dimensão fractal e quantificação de colágeno total, incluindo as frações dos tipos I e III. O impacto da exposição à mistura de DEs durante os períodos fetal e lactacional foi avaliado por meio do teste T de Student, considerando diferenças significativas quando $P \leq 0,05$. Os resultados indicam que a composição de desreguladores endócrinos resultou em aumento da proporção de fibras de colágeno dos tipos I e III, redução no número de folículos e corpos lúteos, além de diminuição nos níveis séricos de 17β-Estradiol e progesterona nas fêmeas da descendência F1. Coletivamente, essas descobertas ressaltam que a exposição multigeracional está associada à fibrose ovariana em ratas da geração F1, comprometendo o desenvolvimento de folículos antrais, a capacidade ovulatória e a formação de corpos lúteos.

Palavras-chave: foliculogênese, ovário, DOHaD, colágeno, matriz extracelular, corpos-lúteos, fibrose.

ABSTRACT

Impact of perinatal exposure to environmentally relevant endocrine disruptors on ovarian folliculogenesis and remodeling in adult rats

Endocrine disruptors (EDs) are chemical substances ubiquitous in the human and animal environment that can disrupt the endocrine system and affect reproductive processes such as ovarian folliculogenesis. In this study, we investigated the effects of a mixture of endocrine disruptors (ED -mix) based on human environmental exposure on ovarian folliculogenesis and ovarian tissue organization in adult rats whose mothers were exposed to this mixture during gestation and lactation. To achieve this objective, pregnant females of the Sprague-Dawley line were randomly divided into two experimental groups: (i) the control group (Control), which received an oral administration of 2ml/kg corn oil, and (ii) the ED mix group, which received an oral administration of 32.11mg/kg/day of a mixture of twelve compounds (phthalates, pesticides, UV filters, bisphenol A, butylparaben) diluted in 2ml/kg corn oil Pregnant and lactating rats were treated from gestational day 7 (GD7) to postnatal day 21 (DPN21). After weaning, on PND22, there was a pause in the induction of mixtures of these compounds, and the females of the F1 offspring were sacrificed on postnatal day 360. The ovaries were removed, dissected, and examined histologically. Blood samples were also taken from the animals to determine hormone levels of estradiol and progesterone. Hematoxylin-eosin and picrosirius-stained tissue sections were examined for morphologic characteristics, measurement of fractal dimension, and quantification of total collagen, including type I and III fractions. The effects of exposure to the ED mixture during the fetal and lactation periods were assessed by Student's T test, with differences considered significant at $P \leq 0.05$. The results indicate that the mixture of endocrine disruptors led to an increase in the proportion of type I collagen fibers and III, a decrease in the number of follicles and corpora lutea, and a decrease in serum levels of 17β-estradiol and progesterone in F1-generation females. Overall, these results highlight that multigenerational exposure contributes to ovarian fibrosis in F1 generation rats and impairs antral follicle development, ovulatory capacity, and corpus luteum formation.

Keywords: folliculogenesis, ovary, DOHaD, collagen, extracellular matrix, corpora lutea, fibrosis.

LISTA DE SIGLAS

4-MBC - 4-methyl-benzidylene

CL - Corpus luteum

DBP - Di-n-butyl phthalate

DDT – Dichlorodiphenyltrichloroethane

DE – Desregulador endócrino

DEHP - Diethylhexyl phthalate

DG – Dia de gestação

DOHaD - Developmental Origins of Health and Disease

DPN – Dia pós-natal

ECM - Extracellular matrix

ED - Endocrine-disrupting chemical

FD – Fractal dimension

GD – Gestational day

HE – hematoxylin-eosin

OMC - Octyl methoxynamate

p,p'-DDE - Dichlorodiphenyl-dichloroethylene

PND – Postnatal day

PSR - Picrosirius Red Staining

WHO - World Health Organization

SUMÁRIO

1 ARTIGO CIENTÍFICO

Multigenerational exposure to a mixture of environmental endocrine disruptors impairs follicular development and leads to ovarian fibrosis in adult rats

Giovana De Santi Phelippe Nunes[1], Gabriel Rodrigues Leal de Oliveira[1], Karianne Delalibera Hinokuma[1], Sarah Gomes Nunes[2], Leonardo de Oliveira Mendes[1], Anthony César de Souza Castilho[1*]

Short Title: Environmental endocrine disruptors and ovarian fibrosis. .

[1] University of Western São Paulo (Unoeste), Presidente Prudente, SP, Brazil.

[2] São Paulo State University (Unesp), Department of Pharmacology, Institute of Biosciences, Botucatu, SP, Brazil.

* Corresponding author: University of Western São Paulo, Rodovia Raposo Tavares, km 572, Bairro Limoeiro CEP, 19067-175, Presidente Prudente, SP, Brazil. E-mail address: castilho.anthony@gmail.com

ABSTRACT

Endocrine disruptors (EDs) are chemical substances found in the human and animal environment that can potentially interfere with the endocrine system by disrupting important reproductive processes such as ovarian folliculogenesis. This study aims to investigate the effects of a mixture of endocrine disrupting chemicals (ED-Mix), reflecting the complexity of human environmental exposures, on ovarian follicular mechanisms and ovarian tissue structural integrity in adult female rats derived from dams exposed to the mixture during both pregnancy and lactation. Pregnant Sprague-Dawley rats were intentionally assigned to two different experimental groups: The control group, which was given corn oil by gavage, and the ED mix group, which was given a carefully formulated mixture of twelve different compounds, including phthalates, pesticides, UV filters, bisphenol A and butylparaben – all carefully diluted in corn oil and administered by gavage since gestational day 7 (GD7) until postnatal day 22 (PND22). After weaning, the female F1 offspring were euthanized on day 360. The ovaries were carefully harvested, dissected, and then subjected to rigorous histological examination. Through the use of hematoxylin-eosin and picrosirius staining techniques, we performed a comprehensive analysis that included morphological features, fractal dimensions, and accurate quantification of the different types of collagen. Blood samples were used to determine hormone levels of estradiol and progesterone. The comprehensive results of this study indisputably show that the mixture of endocrine disruptors contributed to a detectable increase in type I and type III collagen fibers, which was accompanied by a remarkable decrease in the quantification of ovarian follicles and corpora lutea. The morphologic findings were accompanied by a decrease in estradiol and progesterone

levels. Taken together, these findings demonstrate that multigenerational exposure to ED mixture induces ovarian fibrosis in F1-generation female rats and disrupts of ovarian hormone secretion.

Key-words: folliculogenesis, *corpora lutea*, DOHaD, ovary, collagen, extracellular matrix.

INTRODUCTION

For a long time, humans have been trying to establish a link between toxic exposure and its biological effects on the body. Endocrine disrupting chemicals (ED) have been identified as substances that can potentially affect human development (Jacobson-Dickman et al., 2009) and can be transmitted through food, air, water, or the environment (Valadares and Pfeilsticker, 2012). They are used by various industries such as herbicides, pesticides, solvents, industrial lubricants, plastics, and plasticizers such as bisphenol A and phthalates (Valadares and Pfeilsticker, 2012). Certain EDs mimic the action of natural hormones by activating their specific receptors, while others bind to hormone receptors and block the action of natural hormones. They can also interfere with the production, transport, metabolism, and excretion of naturally occurring hormones (Cooper et al., 2000).

One of the first obstacles in researching these synthetic compounds is fragmentation of exposure, where researchers tend to focus on isolated or categorized analysis of compounds (Rappaport & Smith, 2010). Rather than assessing individual or groups of two to three factors over short time periods, the goal of this approach was to analyze the greatest number of them simultaneously, despite the greater complexity of experimental design and methods required to achieve the goals (Holland, 2017). Christiansen et al. (2012) created a mixture of

twelve chemical compounds, selected as representative of several others ED, to simulate human exposure. The composition of the mixture consists of 2 phthalates: Di-n-butyl phthalate (DBP) and diethylhexyl phthalate (DEHP); 5 pesticides: vinclozine, prochloraz, procymidone, linuron, epoxiconazole; the metabolite of the pesticide dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (p,p'-DDE). These 8 compounds are characterized by predominantly antiandrogenic properties. In addition, the mixture also contains 4 predominantly estrogenic substances: two UV filters, octyl methoxynamate (OMC) and 4-methylbenzidylene camphor (4-MBC); the phenolic compound BPA; butylparaben, an antifungal preservative (Christiansen et al., 2012; Axelstad et al., 2014; Boberg et al., 2016).The ovary is a central female reproductive organ essential for fertility and normal hormone production. It is a complex organ that undergoes constant structural and functional changes throughout the reproductive lifespan of women (Monget et al., 2021). Folliculogenesis is a sequence of processes that take place in the cortex of the ovary and have the function of differentiating germ cells, first giving rise to oogonia, then oocyte I and the formation of preantral follicles and later antral follicles (Santos et al., 2013). This process can be influenced and affected by several factors, including ED (Karwacka et al. 2019). Therefore, it is necessary to explore the possible effects of this exposure, not only on the exposed individual, but also across generations.

The entire process of folliculogenesis is also mediated by the extracellular matrix (ECM), which is essential for follicular development and provides cell migration, differentiation, division, and adhesion, as well as ovarian tissue strength and elasticity. The extensive composition of extracellular matrix molecules mainly includes proteins such as collagen type 1 and type 3, elastin, integrin and fibronectin.

Thus, the ECM influences the fluid dynamics of the ovarian tissue by remodeling the follicular wall according to the development and expansion of the follicle and adapting the ovarian microenvironment for reproduction. (Rodgers; Irving-Rodgers; Russell, 2003). Based on human exposure to various agents that can alter homeostasis, characterization of the long-term effects of endocrine disruptors on ovarian function allows identification of potential changes not only in the mother but also in the offspring. Many studies have been published on ED and its impact on folliculogenesis, but research on F1 generation folliculogenesis with progenitors exposed to a mixture of ED is still sparse (Zhou, Gao, and Flaws 2017). Therefore, the present study aims to test the multigenerational effect of a mixture of ED based on human exposure on folliculogenesis. ovarian tissue organization and ovarian steroid levels of F1 offspring.

MATERIAL AND METHODS

Animals

Twenty-four adult females 120 days of age and 12 adult males 90 days of age were used, both from the Sprague-Dawley line weighing approximately 300 g, acquired at the Multidisciplinary Center for Biological Investigations in Laboratory Animal Science (CEMIB/UNICAMP). The animals were kept in the small mammal facility of the Universidade do Oeste Paulista (Unoeste) under controlled light (12 hours of light/12 hours of darkness) and temperature conditions (average 23ºC to 25ºC) They were fed a commercial phytoestrogen-free diet (NUVILABCR1/NuvitalPR) and filtered water in glass troughs with lids and metal

spouts with a capacity of 500 ml. Water and feed were provided ad libitum. Animals were housed in a ratio of two to three females and one adult male for mating in a polypropylene cage measuring 41x34x16 cm with a stainless steel lid in the shape of a grid and lined with autoclaved white pine shavings, and the cage and shavings were replaced twice a week. Relative humidity (55g/m³ ± 10g/m³) and continuous exhaust air were controlled throughout the experimental period. Animal handling, drug administration, stunning, and euthanasia were submitted to the Ethics Committee for the Use of Animals (Protocol number: 7576).

ED-Mixture

Endocrine disruptors: Vinclozine (Cas nº 50-471-44-8), DEHP (Cas nº 117-81-7), DBP (Cas nº 175606-05-0), prochloraz (Cas nº 67747-09-5), epoxiconazole (Cas nº 133855-98-8), p,p'-DDE (Cas nº 72-55-9), Procymidone (Cas nº 32809-16-8), Linuron (Cas nº 330-55-2), BPA (Cas nº 80-05-07), Butylparaben (Cas nº 94-26-8), 4-MBC (Cas nº 36861-47-9) and OMC (Cas nº 5466-77-3), were purchased from Sigma-Aldrich.

Experimental design

Mating occurred during the dark phase of the cycle, with two to three females placed in the male's box. Day of gestation/gestation (DG0) was determined by the presence of sperm in the vaginal swab and positive vaginal cytology in the estrus phase. Females identified as pregnant were individually caged and randomly divided into 2 experimental groups (n=10/group): ED Mix group: 32.11 mg/kg/day ED Mix diluted in corn oil (2 ml/kg); Control group: (vehicle: 2 ml/kg corn oil, by gavage). Animals in the ED Mix group received a ED mix developed by Christiansen et al.

(2012) and reproduced by Axelstad et al. (2014), Isling et al. (2014), Boberg et al. (2015), Mandrup et al. (2014), and Johansson et al. (2016). The composition of the mixtures was determined based on the adjusted human intake (mg/kg body weight/day) and chosen as the basis for the study of the mixtures of each ED and multiplied by 100 due to the accelerated metabolism of mice. DBP (0.01), DEHP (0.02), vinclozine (0.009), prochloraz (0.014), procymidone (0.015), linuron (0.0006), epoxiconazole (0.01), p,p'-DDE (0.001), 4-MBC (0.06), OMC (0.12), bisphenol A (0.0015), and butylparaben (0.06) multiplied by 100 resulted in a mixture of 32.11 mg/kg body weight per day (Christiansen et al. 2012). In this procedure, the mixture ED was adjusted compared to the original, with acetaminophen removed from the composition due to the hepatotoxic potential of the drug, making daily and prolonged exposure impossible.

The mixture ED was administered to pregnant/lactating rats from gestational day 7 (DG7) to postnatal day 21 (DPN21) at the same time points (8h to 10h). Females were kept in individual cages and weighed every other day to calculate the volume of the mixture ED. After birth, the number of pups per litter was adjusted to 8 per litter (4 females and 4 males) to maintain a 1:1 male-to-female ratio, and litters with fewer than 7 pups were excluded from the search. On day 360, females were euthanized by intramuscular injection of an overdose of the anesthetics ketamine and xylazine.

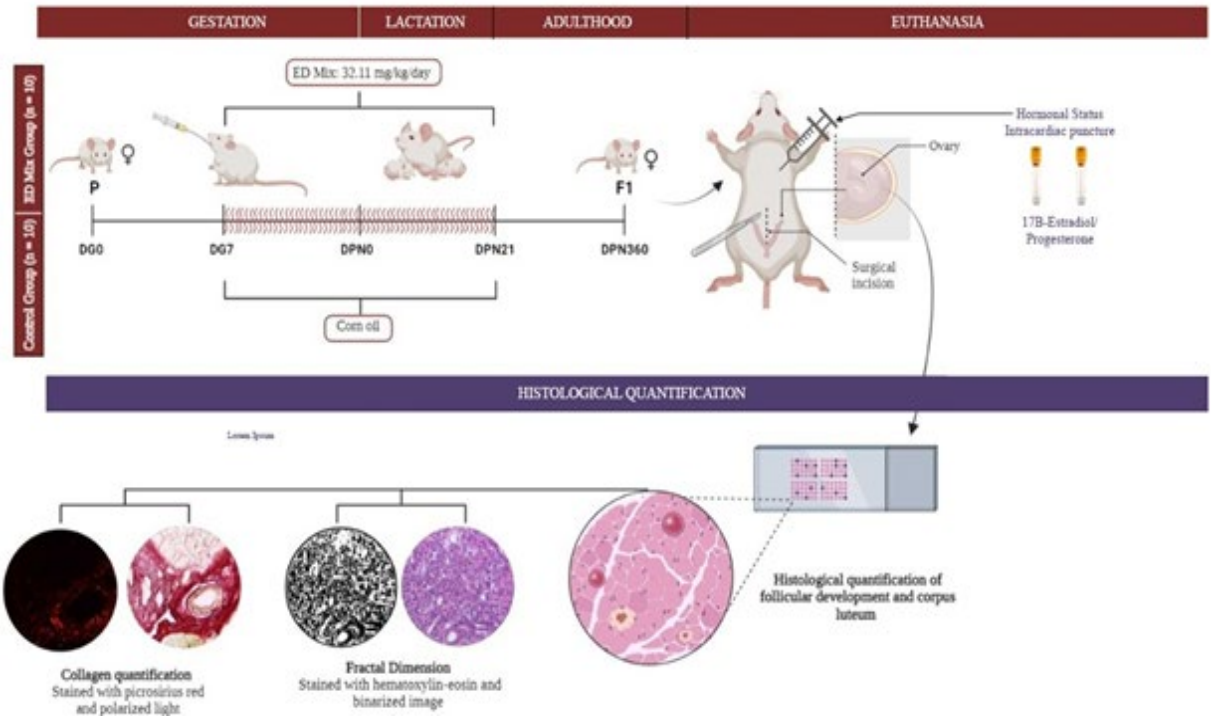

Figure 1. Experimental design. The lines refer to the period of exposure of pregnant rats (GD07 to PND21) with the 12 ED mixture. GD (Gestational Day); PND (Postnatal Day).

Ovary assessment

The weight of the rats was determined immediately after intraperitoneal injection of the anesthetics; they were then euthanized. The weight of each ovary was determined, and the organ coefficient was calculated as follows: Organ weight/body weight × 100%. The organosomatic parameter was used to evaluate animal welfare conditions (Schimitt et al. 2020). To evaluate the effect of a ED mixture on follicular and corpus luteum development, the ovaries were removed at DPN360 for histological quantification of follicular development and corpus luteum formation (CL). After removal, ovaries were weighed individually and fixed in

Metacarn and stored in 70% ethanol. Fragments were then dehydrated by treatment with a series of ethanol solutions of increasing concentrations (80%, 90%, 95%, and 100%), clarified in xylene, and embedded in Paraplast (Oxford Labware, St. Louis, MO, USA). Sections 4 µm thick were prepared and stained with hematoxylin and eosin (HE) and picrosirius red (PSR). Images were acquired using an Axiophot II digital photomicroscope (Zeiss Jenaval, Jena, Germany8) as described in the following sections. Samples stained with HE were used for morphometric and FD analysis, whereas PSR staining was used for quantification of total collagen, collagen types, and FD analysis. For quantification, 10 sections of the entire ovary were digitized using an automated digital microscope. Antral follicles and CLs were manually quantified by an experimenter blinded to treatment. The total area of all visible antral follicles and CLs was measured using ImageJ software (National Institute of Health, United States - NIH), which is available free of charge online (https://imagej.net/ij/index.html).

Fractal Dimension

The H&E-stained histological sections were analyzed in 10 animals per group, photographed (10 histological fields/section, 400× magnification), and binarized for reading the fractal dimension. Fractal dimension (FD) is used to quantify structural tissue changes. It is a useful technique for quantifying organization in an image using fractals that describe the extent of space and self-similarity of structure (Frisch et al., 2012). To perform the fractal analysis with the aim of distinguishing the tissue in the slides HE and PSR, 10 histological sections of each group (control; ED mix) were used, with 5 histological fields photographed per slide (40× magnification),

distinguished by the fact that the structures did not overlap. They were then analyzed in ImageJ software (National Institute of Health, United States - NIH), available free of charge on the Internet (http://rsbweb.nih.gov/ij/), using the box-counting method. These photographs are then binarized in two dimensions so that the pixels can be quantified, the tissue separated as a whole, and the fine structures present in the section determined statistically.

Histological fractal assessment is based on the relationship between the resolution and the assessed scale. The result was quantitatively expressed as the fractal dimension of the object, which is DF= (Log Nr / log r-1), where Nr is the number of equal elements needed to fill the original object and r is the scale applied to the object, so that the statistics of the fractal dimension, which are always between 0 and 2, are short numbers that leave the similarities between the closest structures, do not evaluate different textures.

Collagen quantification

For samples stained with PSR, analysis was performed using ImageJ, following the software instructions for quantification of collagen. The threshold method was used to quantify collagen. Images were acquired using an Axiophot II digital photomicroscope (Zeiss Jenaval, Jena, Germany) with a 40x objective. Ten images were analyzed per sample. ImageJ analysis provides the percentage of pixels in this image (area fraction), which represents the percentage of collagen/area. In addition, the same samples (PSR staining) were also analyzed with polarized light, distinguishing collagen types based on the intensity of the birefringent fibers. The slides were analyzed with polarized light, which facilitates the differentiation of collagen types based on the intensity of the birefringent fibers: red and green

represent collagen types I and III, respectively (Junqueira et al. 1978; Montes et al. 1980). Collagen fibers were differentiated and quantified using the RGB Measure tool from ImageJ. This method allows the red, green, and blue channels of an RGB image to be measured separately (ImageJ, 2004). Therefore, in our work, we used only the quantifications of the red and green channels, which represent the percentage of collagen type I and III, respectively. These values were used for statistical analysis

Hormonal status

Blood samples were collected by intracardiac puncture at the time of sacrifice. Serum was separated by centrifugation, aliquoted, and stored at −20°C until analysis. Samples were assigned a sequential code number, and assays were performed in a blinded fashion. 17β-Estradiol and progesterone were determined using a commercial immunoassay (Biomérieux France). Kits were used according to the manufacturer's instructions based on the enzyme-linked fluorescence assay (ELFA).

Statistical analysis

The data obtained were statistically tested using the Shapiro-Wilk normality test. The effects of EDC mixture on folliculogenesis, ovarian tissue remodeling, and hormonal profile were compared using Student's t-test. Data were log-transformed to fit the normal distribution, and all data showed a normal distribution. Analyzes were performed using JMP software (SAS Institute Cary, NC, USA). Data are presented as means ± SEM. Differences were considered significant when $p < 0.05$.

RESULTS

Overall, ED -mix exposure was able to decrease the number of antral follicles (p=0.0094) in female F1 progeny. Similarly, ED -mix exposure also decreased the formation of the corpus luteum (p=0.03). However, corpus luteum area (p=0.29; Figure 2) and antral follicles were not affected by EDs exposure (p=0.29; Table 1). Regarding the general phenotypes, we found that ED -mix exposure did not affect the somatic index of the organs (right and left ovary; p=0.61). As for ovarian remodeling, we found no effect of ED -mix on ovarian organization in the present study (p > 0.05, Figure 2). Nevertheless, using PSR staining, we found a higher level of FD in the ovaries of female F1 exposed to ED -mix (Figure 3). In collagen analysis, we also observed that ED exposure increased total collagen (p < 0.001, Figure 2A) and induced higher levels of type I collagens (p=0.0095) and III (p < 0.001, Figure 3). Regarding the hormone dose profile, 17β-estradiol (p=0.02) and progesterone (p=0.04) levels were lower in the ED -mix group (table 1). Table 1. Effects of ED mixture on antral folliculogenesis, corpus luteum development, and hormonal status. Data are presented as means ± SEM. Differences were considered significant when P<0.05. Different letters (a;b) indicate significant differences**.**

Parameters	**Control Group**[a]	**ED-Mix**
Antral Follicle (Area -μm^2)	217084 ± 46826	145915 ± 44951
Antral Follicle Count	**3,7 ± 0,68** [a]	**1,3 ± 0,3** [b]
Corpus luteum (Area -μm^2)	411638 ± 85613	286644 ± 75791
Corpus luteum Count	**7,3 ± 1,1** [a]	**4,5 ± 1,1** [b]
Right Ovary (g)	2,05 ± 0,28	2,25 ± 0,29
Left Ovary (g)	2,26 ± 0,17	2,35 ± 0,23
17β-Estradiol (pg/ml)	**34 ± 12** [a]	**28,7 ± 2** [b]
Progesterone (ng/ml)	**28 ± 0,9** [a]	**21,9 ± 1,2** [b]

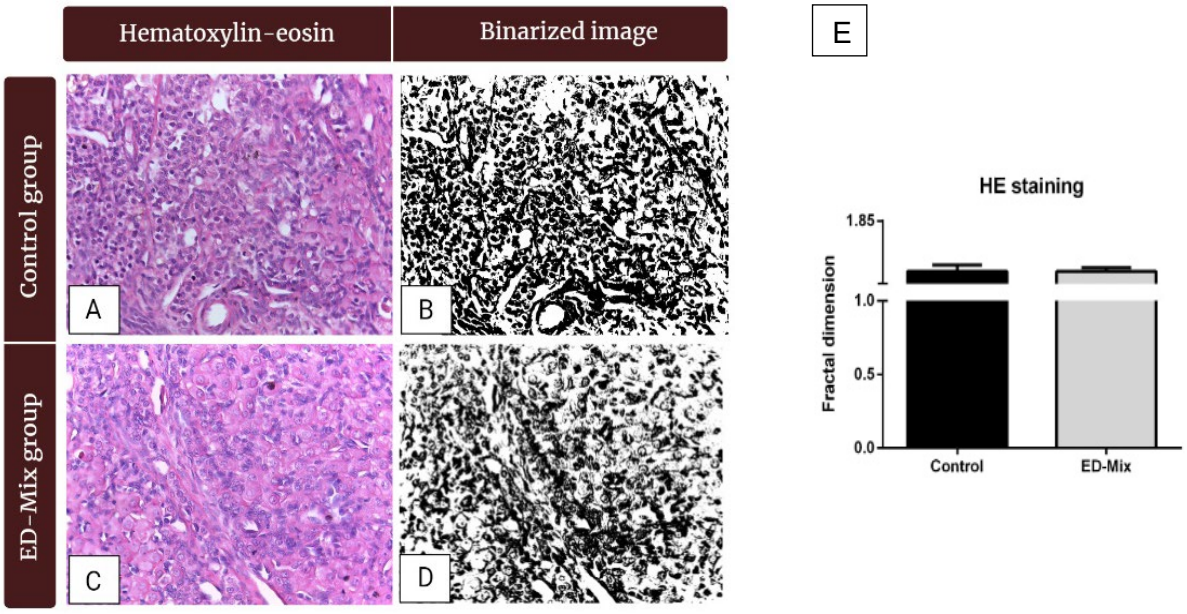

Figure 2. Histological sections of the ovary stained with hematoxylin-eosin in the control group (panel A) and the ED-Mix group (panel C). Plots of photographs B and D, binarized, using the fractal dimension method in ImageJ software. Graphical representation (panel E) of the fractal dimension. Data are given as means ± SEM. No effects of ED-mixing were detected (P>0.05). Magnification: 400X. Staining: H&E.

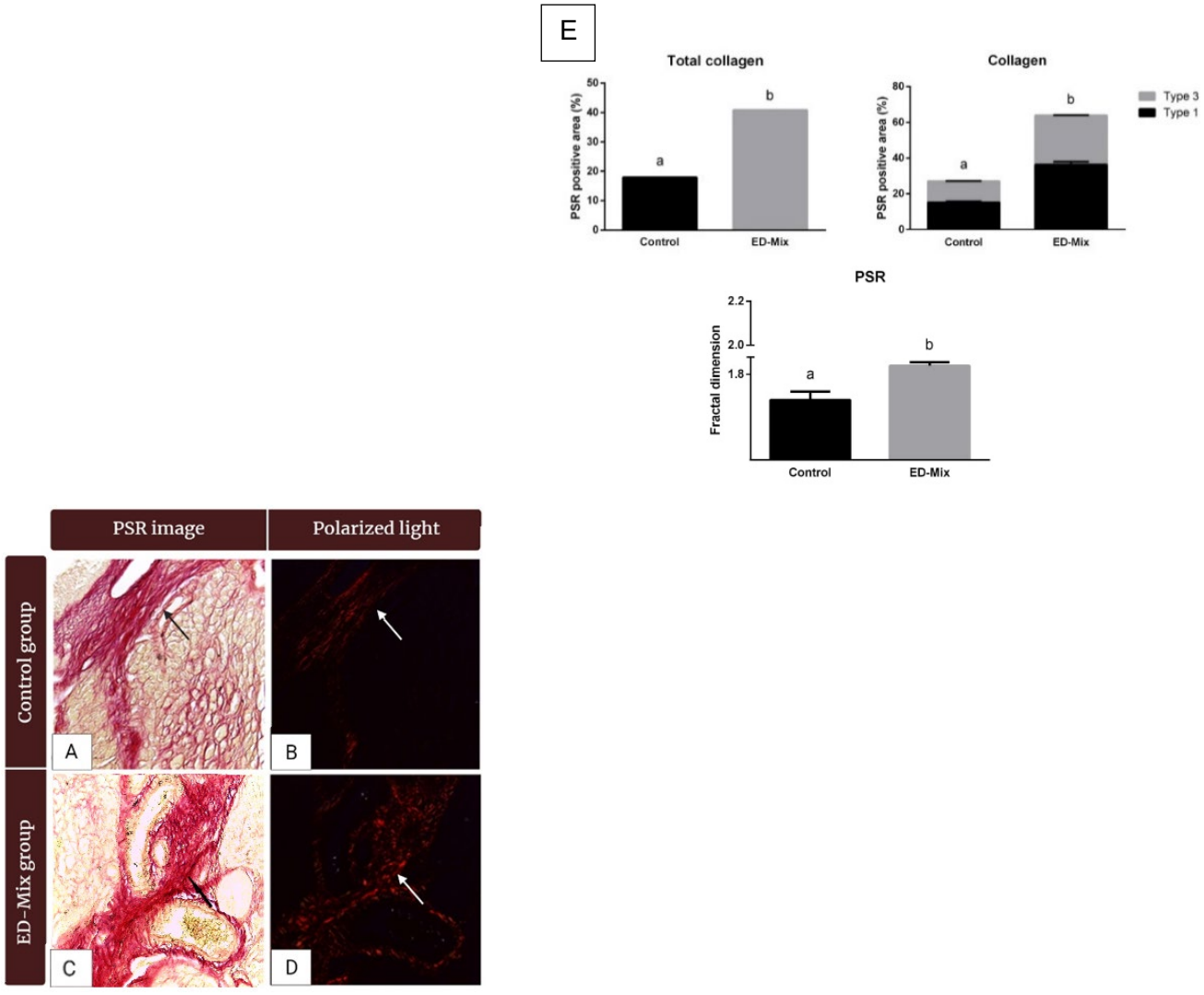

Figure 3. Collagen analysis of ovaries of control group (panel A) and ED -mix group (panel C) stained with picrosirius red. Collagen fibers (arrows) with birefringent intensity in the control group (panel B) and the ED-Mix group (panel D). Graphical representation (panel E) of total collagen area and collagen types I and III and. Data are presented as means ± SEM. Differences were considered significant when $P<0.05$. Different letters (a;b) indicate significant differences. Magnification 400X. Staining: PSR.

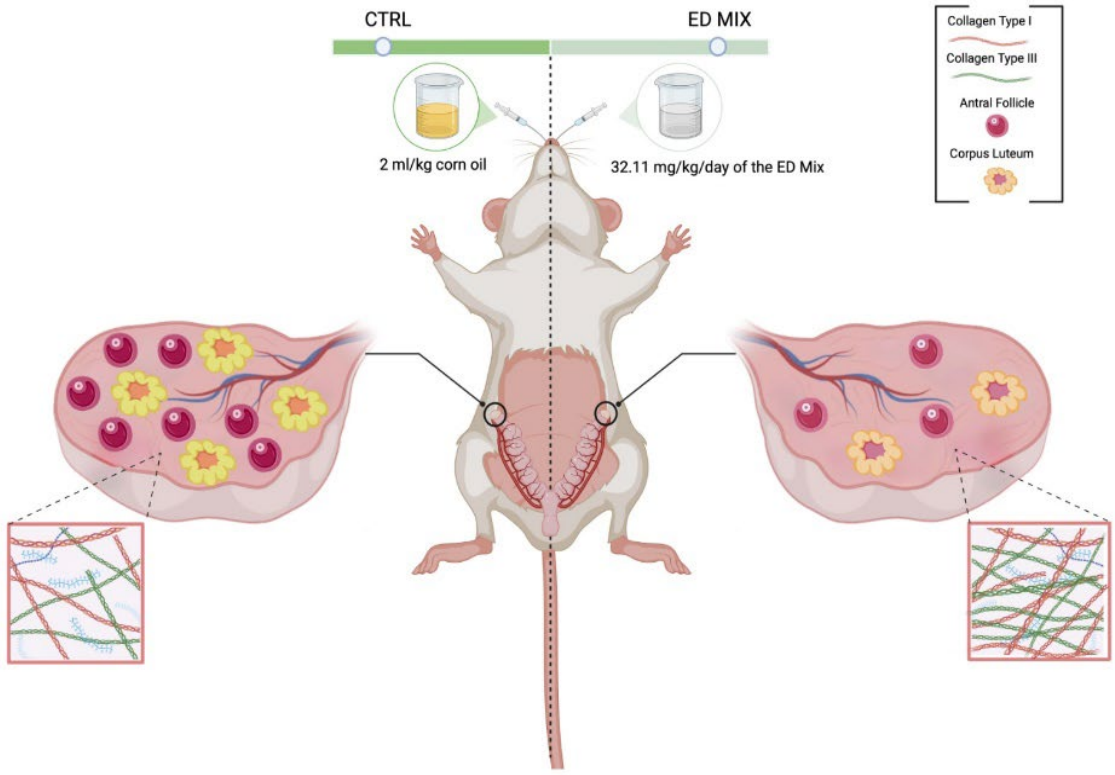

Figure 4. Representative scheme for the finding that perinatal, multigenerational exposure to endocrine disrupting environmental chemicals induces ovarian fibrosis and impairs antral follicle formation and ovulatory capacity of female F1 offspring.

DISCUSSION

Developmental Origins of Health and Disease (DOHaD) postulates a paradigm that environmental influences to which individuals are exposed during embryogenesis increase disease risk throughout life and across generations. In recent decades, more studies on DOHaD have been conducted to shed light on the biotoxic effects of a generation already exposed to chemical compounds in the womb (Suzuki, 2018). Our study highlights the multigenerational ED exposure and further effects on ovarian fibrosis in F1 generation female rats. In summary, the ED mixture was able to affect the development of antral follicles and corpus luteum in female rats

exposed during their perinatal life (Figure 4). Considering this problem, further studies are being conducted in the literature on "DOHaD" and endocrine disruptors associated with causes of infertility. In addition, further studies examining toxicity and intergenerational risk associated with endocrine disruptors are needed to understand the key role of environmental agents in female fertility. In addition, many studies have examined only exposure to individual substances and the resulting disease endpoints. These studies are an important starting point for understanding the mechanisms of action of each ED. Studies examining exposure to multiple substances are also critical because humans are rarely, if ever, exposed to a single environmental pollutant. In this scenario, our findings of impaired antral follicular development and ovulation are demonstrated for several environmental EDs.

According to Rattan and Flaws (2019), endocrine disruptors affect the interplay between the individual organs of the hypothalamic-pituitary-ovarian axis, which is responsible for the adequacy of the female reproductive system. They can disrupt the development of follicles at different stages by retracting folliculogenesis from the beginning of the process, which can lead to atresia, a decrease in the number of these follicles, misdirection, and follicle formation. This loss of functionality or number of follicles favors the decline of the cell assemblies responsible for the production of estrogens, such as the corpora lutea. Thus, the decline in hormone production leads to dysregulation of the hypothalamic-pituitary-ovarian axis, which alters the estrus or menstrual cycle by reducing the induction of LH and FSH, which are responsible for the sufficient number of follicles for ovulation. (Hannon and Flaws, 2015; Rattan and Flaws 2019). In addition, Johansson et al. (2016) showed that exposure to a mixture of endocrine disruptors at levels based on the human exposome during the perinatal period in pregnant female rats leads to a decrease in

ovarian follicular reserve in the offspring, consequently a decrease in the number of follicles and corpora lutea, deregulating external cycles and leading to early senescence; these results are compatible with those of the present study, which links such substances to the imbalance of female reproductive function.

In addition, exposure to ED mix also impaired 17β-estradiol and progesterone. The 17β-estradiol is a hormone secreted in the granulosa cells of the developing antral follicle. This steroid hormone is associated with several phases of the menstrual cycle, particularly the follicular phase, acts in the antral follicle property associated with folliculogenesis, in the control of FSH and LH immunity, and is the hormone responsible for endometrial preparation (McNatty et al., 1979; Amsterdam et al., 1999). Progesterone is a steroid hormone produced in the corpus luteum. Its serum levels are important for the phases of the menstrual cycle, the receptivity of the endometrium to fertilization, the maintenance of pregnancy, and are also responsible for female sexual characteristics (Stefanick, 2005; Stouffer 2003). Decreased levels of estradiol and progesterone are associated with ovarian pathologies and dysfunction associated with infertility (Chauvin, Cohen-Tannoudji, and Guigon, 2022), damage to the corpus luteum leading to increased serum progesterone levels is associated with cases of spontaneous abortion (Taraborrelli, 2015). Here, the disturbances in the production and regulation of 17-beta-estradiol and progesterone could also reflect the growth and maintenance of the endometrium during the menstrual cycle, as well as the preparation of the uterus for pregnancy and the maintenance of a favorable environment for embryo implantation. The uterus undergoes dynamic changes during the estrous cycle and pregnancy, largely determined by the interplay of 17-beta estradiol and progesterone. Disruption of the delicate balance between these hormones can have profound effects on uterine

physiology. Decreased 17-beta estradiol levels can lead to inadequate endometrial growth and impaired vascularization, which can interfere with embryo implantation. In addition, decreased progesterone levels may result in the inability of the uterus to maintain a nurturing environment, leading to early pregnancy loss or reduced litter size.

The intricate relationship between extracellular matrix (ECM) remodeling, collagen types I and III, and ovarian function underscores the importance of understanding the role of ECM in reproductive processes. The influence of endocrine disruptors on ovarian ECM dynamics adds complexity to an already complicated regulatory network. Collagen types I to III are proteins in the form of a triple helix that are mainly involved in the healing process of various tissues of the body. In tissue fibrosis, collagen type I predominates, followed by collagen type III (McKleroy, Lee, and Atabai, 2013), which is consistent with the results of the study. Ovarian fibrosis is a condition in which the ovarian tissue has an excessive proliferation of fibroblasts that synthesize the proteins that make up the ECM, so the extracellular matrix also has excessive growth. Histologically, the ovarian tissue in this condition is thick, rigid, and with a lower number of follicles, as in our study. Thus, the acceleration of ECM components leads to an increase in type I collagen and III, resulting in fibrosis of the tissue, which is consistent with our results. This condition is believed to be the main reason for ovarian dysfunction (Zhou, Shi, and Zang, 2017). In conclusion, ED mixture can affect ovarian function by changing hormone signaling, ECM composition, and cell behavior. It has been shown to affect collagen synthesis and ECM remodeling processes in ovaries, which could disrupt the fine-tuned balance between type I and III collagens, affecting ovarian function and fertility.The rise in the number of infertile people in recent years has led to questions and answers about

why the failure to have a child has psychological effects and causes disruption in both women and men (Yilmaz and Kavak, 2018; Ozkan et al., 2015). According to World Health Organization 50 to 80 million people worldwide have problems with infertility. Given this problem, studies focusing on "DOHaD" and endocrine disruptors associated with causes of infertility are accumulating in the literature. Exposure to these chemical compounds, which are presented to us from embryonic development and precede multigenerational and transgenerational diseases, presents a critical warning in their production and disposal. We are gaining a better understanding of how EDs work and whether their effects can be transmitted across multiple generations. The exposure to these chemical compounds that we have been exposed to since embryonic development and that precedes the development of multigenerational and transgenerational diseases makes it clear that their manufacture and disposal is a critical issue. Public policies to improve the quality of life should consider the production of less harmful chemical compounds and the proper disposal of these chemicals, because we know that they hover over our environment. It is imperative that these findings lead to a reassessment of our approach to protecting reproductive well-being, not only for today's population, but also for the legacy we leave for future generations

CONCLUSION

In summary, the comprehensive investigation of multigenerational exposure to a mixture of endocrine disruptors has illuminated a worrisome panorama of reproductive health. The intricate web of effects has shown that antral follicle formation and corpus luteum development, central processes in the female reproductive cycle, are clearly impaired. As a direct result, the once finely tuned

balance of serum levels of 17β-estradiol and progesterone is disrupted, casting a shadow over the intricate hormonal orchestration that underpins fertility and reproductive vitality. The occurrence of fibrosis in the ovaries as a result of this exposure adds another complex layer to the effects, reflecting the profound impact of these disruptors on ovarian microarchitecture. In essence, this study serves as an alarming reminder of the far-reaching effects that endocrine disruptors can exert across generations and underscores the urgency of stricter regulatory measures and greater awareness of these ubiquitous substances.

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ANEXO A- NORMAS DA REVISTA ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH

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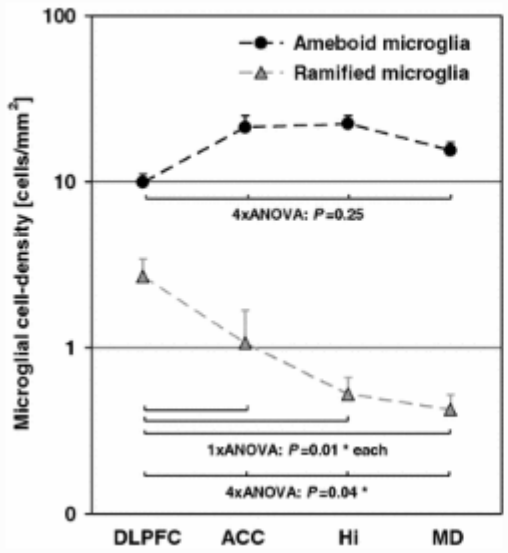

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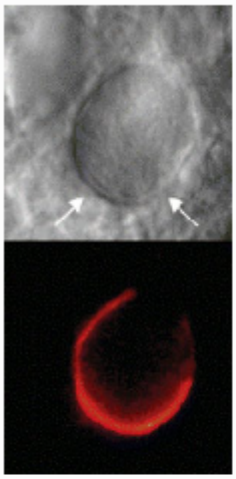

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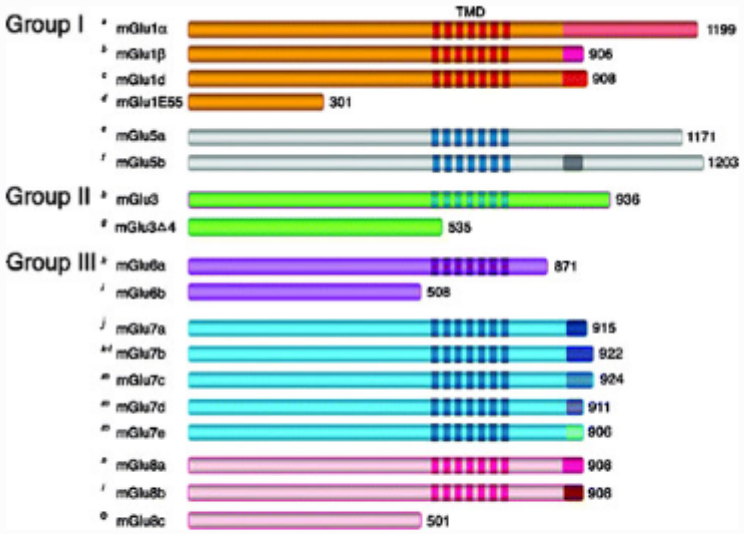

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