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Neonatal exposure to estradiol valerate reprograms the rat ovary androgen receptor and anti-Müllerian hormone to a polycystic ovary phenotype

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ABSTRACT

To understand the impact of exposure to steroids in the early step of ovary development (a stage occurring *in uterus* in humans), we studied neonatal exposure to estradiol valerate (EV) in rats regarding polycystic ovary (PCO) development as well as expression of androgen receptor (Ar) and anti-Müllerian hormone (AMH), a marker of ovarian follicular development. Rats exposed to one dose of EV (10 mg/kg, sc) during their first 12 h of life were euthanized at 2, 30 and 60 days of age. Gene array and real-time PCR studies showed *Ar* and *AMH* up regulation in the ovary at 2 days of age and persisted at 60 days of age, when a PCO phenotype was evident with increased levels of Ar and AMH proteins. The single neonatal exposure in rats suggests participation of EV in developing PCO syndrome. Its persistence also suggests that estradiol reprograms ovarian function and disease during adulthood.

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1. Introduction

In mammals, ovarian folliculogenesis starts with the formation of primordial follicles, following oocyte nest breakdown, allowing the oocytes to be surrounded by a layer of somatic cells and form individual primordial follicles in a process known as follicular assembly and undergo the initial transition from the primordial to primary follicular stage [1–3]. These processes are controlled by multiple regulatory factors among which estradiol is one of the most important [4,5], making this process vulnerable to hormonal changes induced by external factors. Environmental endocrine disruptors mimicking estrogenic compounds cause detrimental effects on these early ovarian processes. Compared with ovarian development in humans, the ovarian primary follicle population in the rat is not reached until shortly after birth because is an immature mammal. Thus, the neonatal rat is a good model to study early ovarian development without the contribution of the multitude of factors regulating pregnancy during the last trimester of pregnancy in humans. [2,3].

One of the most frequent ovarian pathologies in women during their reproductive years is the polycystic ovary syndrome (PCOS). In humans, PCOS is a complex endocrine disorder characterized by hyperandrogenism, ovulatory/menstrual irregularity, and polycystic ovaries [6]. Interestingly, women with PCOS exhibit a significant increase in androgen concentrations during pregnancy [7]. An important proportion of the first-degree female relatives of women with PCOS have been shown to be at risk for developing PCOS [8], suggesting a reprogramming factor that affects key hormones or receptors controlling ovary function and, hence, follicular development. In fact, compared with control prepubertal girls, daughters of PCOS women exhibit higher levels of anti-Müllerian hormone (AMH), a marker of growing follicles, beginning at the peripubertal stage [9,10]. It has been proposed that this inheritance is not the result of a genetic condition but is due to fetal programming [6,11].

A form of PCO resembling some aspects of human PCOS can be induced in rats by a single injection of estradiol valerate. This model has been widely used to study PCO and the metabolic effects of hormone expression that resemble human PCOS such as oligo/anovulation, follicular cysts, and insulin resistance [12–23].

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Although the steroid-induced PCO model in rats lacks some characteristics of women with PCOS, such as high androgen plasma levels, the ovary presents a high androgen and estradiol secretory and biosynthetic capacity [15,24]. Brawer et al. [19,20] demonstrated that administering estradiol to adult rats induces the polycystic condition 60 days after hormone exposure. Subsequently, Rosa-e-Silva et al. [15] and Sotomayor-Zárate et al. [16] verified that the same occurred in rats that were injected with EV at 14 days of age and in the first day after birth, respectively. In addition, our laboratory demonstrated a temporal window in which the administration of EV induces irreversible damage to folliculogenesis, ovulation, and the reproductive physiology, distinguishing the neonatal stage as a vulnerable period [13]. Regarding the fetal exposure to steroids as a factor to modify ovary function, it has also been reported that the exposure of pregnant sheep to testosterone or the nonaromatizable androgen dihydrotestosterone (DHT) produced differential effects on the reproductive function of progeny. Increased numbers of growing follicles and reduced numbers of primordial follicles were found in 10-month-old prenatal testosterone-treated but not DHT-treated female sheep, suggesting that increased follicular recruitment of prenatal testosterone-treated female sheep is facilitated by androgenic programming but that postpubertal follicular growth, antral follicular disruptions, and follicular depletion are mediated largely via estrogenic reprogramming [25,26].

These results suggest that the exposure of rats to EV during the neonatal period acts to determine ovarian function during development in a similar way as that occurs in humans and sheep, in which ovary development occurs *in uterus* during development for both mammals. Thus, we analyzed whether neonatal exposure to estradiol changes the genes that regulate ovarian morphology and function and whether these changes persist during development.

2. Materials and methods

2.1. Animals and experimental design

Sprague- Dawley rats weighing 250–300 g were maintained at 20 °C with a 12-h light and 12-h dark cycle. Water and food were available *ad libitum*. The estrous cycling activity of the rats was monitored with daily vaginal smears. On an afternoon of proestrus, rats were mated, and pregnancy was confirmed the following morning by checking for a vaginal plug. From the pups obtained from these pregnancies, 36 females were randomly assigned to the control group (n = 18) or estradiol valerate (EV) group (n = 18). A single dose of estradiol valerate (EV; Sigma, St. Louis, MO, USA) was administered during the first 12 h after birth. The administered dose was 10 mg/kg of body weight dissolved in 50 μL of sesame oil as the vehicle (Sigma, St. Louis, MO, USA) as previously described [16]. The controls were injected with 50 μL of the vehicle.

Females rats were euthanized by decapitation at 2 (24 h after EV administration), 30 or 60 days of age (n = 6 for EV-treated and control rats per age group). These ages were used because they represent different stages of reproductive development in rats, and, hence, different stages of follicular development. The ovaries were isolated, the left ovary was frozen at −80 °C for RNA extraction (2, 30 and 60 days of age) and the right ovary was fixed in Bouin solution for immunohistochemistry (60 days of age). Trunk blood was collected, and plasma was obtained for estradiol measurements.

For the 60 days of age rats, estrous cyclicity was also monitored by analysis of vaginal lavage fluid taken at 10 AM daily to verify the stage of the estrous cycle. Because rats treated with EV at different ages are in persistent estrous [14–16,20], control rats were also euthanized during the estrous stage of the cycle. All experimental procedures were approved by the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences at the University of Chile and complied with national guidelines (CONICYT Guide for the Care and Use of Laboratory Animals).

2.2. Determination of the clearance rate of plasma estradiol valerate and measurements of the plasma levels of estradiol

Another group of 18 control female pups and 23 EV female pups were used to determine the half-life and plasma levels of estradiol. We injected neonatal female rats during the first 12 h after birth with one dose of EV, 10 mg/kg of body weight, in 50 μL of sesame oil as the vehicle. The rats were sacrificed at 2.5 days of age (n = 4 control rats, n = 6 EV rats), at 3.5 days of age (n = 4 controls rats, n = 5 EV rats), at 4.5 days of age (n = 5 controls rats, n = 5 EV rats) and at 15 days of age (n = 5 controls rats, n = 7 EV rats).

At the time of sacrifice, trunk blood was collected from the rats of both groups, and plasma was isolated and used to determine the levels of estradiol using the ELISA kit 11-ESTHU-E01 (ALPCO Diagnostics, Salem, NH, USA). Each sample was run in duplicate. The kit sensitivity was 10 pg/mL with a cross reactivity of less than 1.6% with other steroidal hormones. The intra- and inter-assay variability was less than 10%.

Data from the plasma of the rats of the EV group were used to calculate the EV clearance half-life ($t_{½}$), which was calculated by plotting the ln of the plasma concentrations of estradiol versus time.

2.3. PCR array

To analyze the general modifications in gene expression, a quantitative RT-PCR-based array assay (PCR array) was performed containing 84 genes. To analyze the short-term effects of EV on gene expression, the ovaries from 2 days of age rats were analyzed with a PCR-array kit for nuclear receptors and coregulators (SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. For the long-term effects, a PCR-array kit for growth factors was used with ovaries from 60 days of age rats. Total RNA extraction was performed using RNeasy columns (Qiagen, Hilden, Germany). cDNA was synthesized using the RT^{2}First Strand kit (SABiosciences). The cDNA from the ovaries of 2 days old rat was synthesized from 1 μg of pooled total RNA from 6 ovaries of different rats. For the 60 days of age rats, one ovary was used and pooled from 6 rats. For both ages, the PCR array was repeated twice. The PCR array protocol was performed using an IQ5 real-time thermocycler (BioRad). The results were analyzed using the virtual platform from SABiosciences. It was considered a cut-off threshold when the determined mRNA was upregulated or downregulated at least 2-fold compared with the control value.

2.4. Real-time PCR

Real-time PCR was performed using single ovary samples, each age group comprised 6 controls and 6 EV female rats. Total RNA extraction was performed using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions, which included digestion of DNA by DNAse in the column.

The primers were designed using the Primer Select program (DNASTAR Inc., USA) and the mRNA sequence provided by the PubMed database as the template. The primer specificity was evaluated *in silico* using Primer-BLAST from PubMed (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to determine whether there were amplification byproducts in the rat genome. Table 1 shows the sequences of the designed primers for real-time PCR and GenBank accession number of each gene used as a reference. The PCR reaction mix contained 10 μL of Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Inc., California, USA); 0.25 μM of each 18S primer, 0.15 μM of each *Amh* primer, or 0.1 μM of each *Ar*

Table 1
Sequences of the primers designed for real-time PCR.

Gene	Primer Sequence		Product Length
Amh (NM_012902.1)	fwd	5′-GGCTCGCCCTAACCCTTCAACC-3′	312 bp
	rev	5′- GTCCCCGCAGAGCACGAACC-3′	
Ar (NM_012502.1)	fwd	5′-AATGGGACCTTGGATGGAGAACTA-3′	297 bp
	rev	5′-TCATAACATTTCCGGAGACGACAC-3′	
	rev	5′-GGTTGTACATTGAGGGGGAAGGTC-3′	
18S (NR_046237.1)	fwd	5′-TCAAGAACGAAAGTCGGAGG-3′	489 bp
	rev	5′-GGACATCTAAGGGCATCACA-3′	

The GenBank accession number of each gene used as a reference for the primer designs is indicated in parentheses. bp, base pair; fwd, forward primer; rev, reverse primer.

primer; 2 μL of cDNA, and sterile water for a final volume of 20 μL. PCR reactions were performed using the IQ5 real-time thermocycler (BioRad) under the following conditions: 95 °C for 5 min for 1 cycle; 40 cycles at 95 °C for 15 s, 60 °C (*Ar* and 18S) or 65 °C (*Amh*) for 15 s, and 72 °C for 30 s; 72 °C for 7 min, and ended with a 4 °C maintenance cycle.

2.5. Morphometric analysis

Ovaries previously fixed in Bouin solution were embedded in paraffin, cut into 6-μm sections, and stained with hematoxylin and eosin. Morphometric analyses of whole ovaries were performed as previously described [13,14]. All follicular structures were followed through all the slices used for immunohistochemistry. Preantral follicles correspond to follicles without antral cavity including primary and secondary follicles. Antral follicles were those with more than 3 healthy granulosa cell layers, the antrum and with a clearly visible nucleus of the oocyte; cystic follicles were devoid of oocytes and displayed a large antral cavity, a well-defined thecal cell layer, and a thin (mostly monolayer) granulosa cell compartment containing apparently healthy cells. Corpora lutea are characterized by the higher size, devoid of oocyte and the presence of luteal cells instead of granulosa cells.

2.6. Immunohistochemistry

Immunohistochemistry (IHC) was performed on 6-μm slices from fixed and paraffin-embedded ovaries, taken from 60 days of age rats as previously described [27] with rabbit anti-AR (cat. no. ab74272; Abcam Ltd., Cambridge, UK) diluted 1:100 or goat anti-AMH (cat. no. sc-6886; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500. Biotinylated goat anti-rabbit IgG (cat. no. sc-2040; Santa Cruz Biotechnology) and biotinylated anti-Goat IgG (cat. no. BA-5000; Vector Laboratories, Inc. Burlingame, CA, USA) were used as secondary antibodies for AR and AMH, respectively. After incubating the samples with HRP-streptavidin, the ImmPACT VIP Peroxidase Substrate kit (Vector Laboratories, Inc.) was used to develop the samples. Images were obtained using an Olympus optical microscope (Olympus CX31, Tokyo, Japan) with Micrometrics SE Premium 4 software (ACCU-SCOPE, Inc., Commack, NY, USA). The results were analyzed by measuring the pixel intensities using Integrated Optical Density (IOD) of the Image Pro Plus 6.0 program (Media Cybernetics, Inc., USA). Areas with strong density were compared with background staining adjacent to the area of interest in the same section. Similar sections of at least 4 slides per ovary and 6 ovaries per group were analyzed; the relative contribution of AMH or AR to the total IOD (100%) of the whole ovary slice in the image was calculated for each follicle, differentiating among preantral follicles, antral follicles, and corpora lutea. The results were expressed as the mean ± the standard error of the mean (SEM). Each age group comprised 6 controls and 6 EV female rats

2.7. Statistical analysis

Differences between the control and treatment groups were analyzed with unpaired *t*-test, and a significant difference was established when the p value was less than 0.05. The minimal number of rats used for the experiments was calculated as described in JF Zar et al. (ref). The tests of D'Agostino and Pearson were applied to verify the normal distribution of data. All statistical analyses were performed using GraphPad Prism v5.0 software (GraphPad Software, San Diego, CA)

3. Results

3.1. Plasma clearance rate of estradiol valerate during early neonatal development

To determine the half-life of EV in circulation, we measured the estradiol plasma levels in EV-treated rats at 2, 2.5, 3.5 and 4.5 days

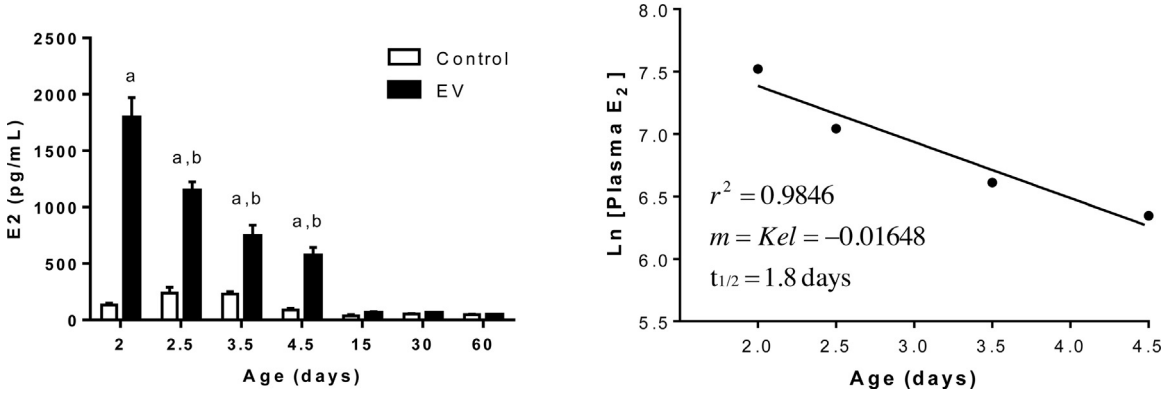
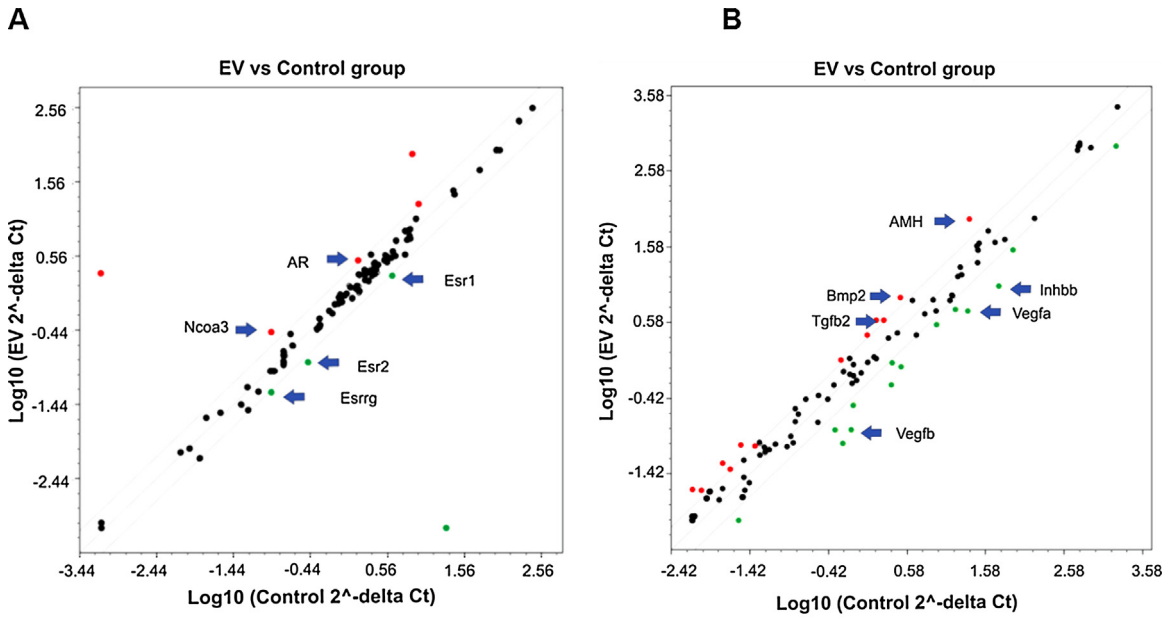


Fig. 1. Half-life of estradiol following injection into neonates. A.- Determination of the elimination kinetics of EV in rats 2, 2.5, 3.5 and 4.5 days of age after measuring the plasma levels of estradiol. B.- Plasma estradiol in controls and EV-treated female rats at different ages. The data are shown as the means ± SEM. The number of rats was 4–5 in the control groups and 5–6 rats in the EV groups. [a] $p < 0.001$ vs control; [b] $p < 0.001$ vs previous age in the same group.



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	Gene Symbol	AVG Ct		2^(-Avg.(Delta(Ct))		Fold Change	Fold up or down Regulation
		Control	EV	Control	EV	(EV/Control)	
2-day old rats	Ar	23.92	23.01	1.49	3.16	2.12	2.12
	Esr1	22.45	23.7	4.13	1.96	0.47	-2.11
	Esr2	26.03	27.6	0.35	0.13	0.38	-2.64
	Esrrg	27.65	28.96	0.11	0.05	0.45	-2.20
	Ncoa3	27.66	26.22	0.11	0.34	3.06	3.06
60-day old rats	Amh	23.3	21.8	23.45	86.79	3.70	3.70
	Bmp10	33.04	35	0.03	0.01	0.34	-2.97
	Bmp2	26.19	25.22	3.16	8.11	2.56	2.56
	Bmp4	28.74	27.96	0.54	1.21	2.25	2.25
	Inhbb	22.06	24.71	55.39	11.55	0.21	-4.80
	Tgfb1	23.87	25.74	15.80	5.65	0.36	-2.79
	Tgfb2	26.91	26.22	1.92	4.05	2.11	2.11
	Vegfb	28.27	31.04	0.75	0.14	0.19	-5.21

Fig. 2. Changes in ovarian gene expression after neonatal estradiol valerate exposure. A.- Nuclear receptor and co-regulator PCR array analysis of mRNA from 2 days of age rats exposed to estradiol valerate (EV); scatter plot showing the fold change of gene expression in EV-exposed rats compared with the controls. B.- Growth factor PCR array analysis for 60 days of age rats exposed to EV; scatter plot showing the fold change in gene expression in the EV-exposed rats relative to the controls. Black circle, unaltered expression; red circle, upregulated gene; green circle, downregulated gene. C.- Summary table of the upregulated (positive values) and downregulated (negative values) genes changed by at least 2-fold relative to the controls in the nuclear receptor or growth factor PCR Array, correspondingly. cDNA from 2 days of age rat ovaries was synthesized from 1 μg of pooled total RNA from 6 different ovaries. For 60 days of age rats, one ovary from each rat was pooled. For both ages, the PCR array was repeated twice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

after injection. The elimination constant for EV obtained in neonatal rats was $K_{el} = -0.01648$; according to the formula $t\,½ = \ln(2)/K_{el}$ [28], the EV half-life was 1.8 days (Fig. 1A). Thus, based on 5 times the half-life, the injected EV would persist in circulation for a maximum of 10 days, which matches with the 13.5-fold increase in E2 found 24 h after exposure to EV, a state that persisted at 4.5 days after EV but was no longer present at 15 days. There were no differences between the controls and treated groups of 15, 30, or 60 days of age rats (Fig. 1B).

3.2. Neonatal exposure to estradiol valerate changes the ovarian gene expression pattern through development

Fig. 2A shows a scatter plot of the fold change in gene expression of EV-exposed 2 days of age rats compared with the controls. The cut-off for up- or downregulation was set at a minimum of a 2-fold change compared with the control values. Five of the analyzed genes showed differential expression in the EV-exposed rat ovaries [Fig. 2A and C; the androgen receptor (*Ar*; overexpressed 2.12-fold), estradiol receptors alpha and beta (*Esr1*, *Esr2*; downreg-

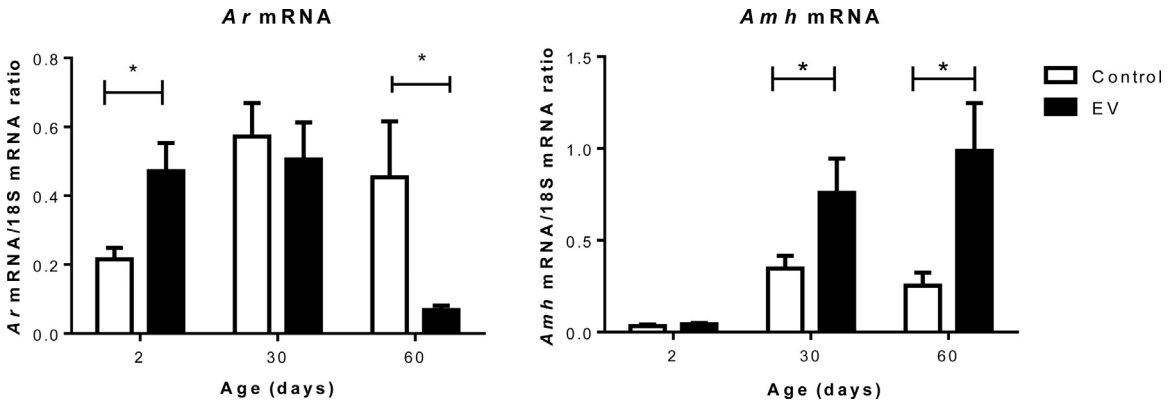

Fig. 3. Ovarian androgen receptor and AMH expression pattern during development after neonatal estradiol exposure. Real-time PCR quantification of A.- Androgen receptor and B.- AMH mRNA levels in the ovaries from 2, 30 and 60 days of age estradiol valerate-treated and control rats. Each age group comprised 6 controls and 6 EV female rats. The data are shown as the means ± SEM. * $p < 0.05$ vs control.

ulated 2.11- and 2.64-fold, respectively), estrogen-related receptor gamma (*Esrrg*; downregulated 2.2-fold), and nuclear receptor coactivator 3 (*Ncoa3*; overexpressed 3.06-fold)]. To determine whether those changes were maintained at 30 and 60 days of age, real-time PCR was performed on peripubertal and adult rat ovaries to analyze the *Ar*-expression pattern during development and determine whether neonatal estradiol exposure can alter *Ar* expression. These ages were used because they represent different stages of reproductive development in rats, and, hence, different stages of follicular development. There were no differences in *Ar* expression between the control and treated 30 days of age rats. By contrast, at 60 days of age, the EV-treated rats had 6.4-fold less *Ar* mRNA than the control rats (Fig. 3A).

Fig. 2B shows a scatter plot of the fold change in growth factor gene expression for EV-exposed rats compared with the controls. Twenty-five genes had altered expression as measured by the mRNA levels (Fig. 2B and C); of these genes, *Amh*, vascular endothelial growth factor, transforming growth factor β (*Tgf-β*), inhibin beta-B, bone morphogenetic proteins (*Bmp*), fibroblast growth fac-

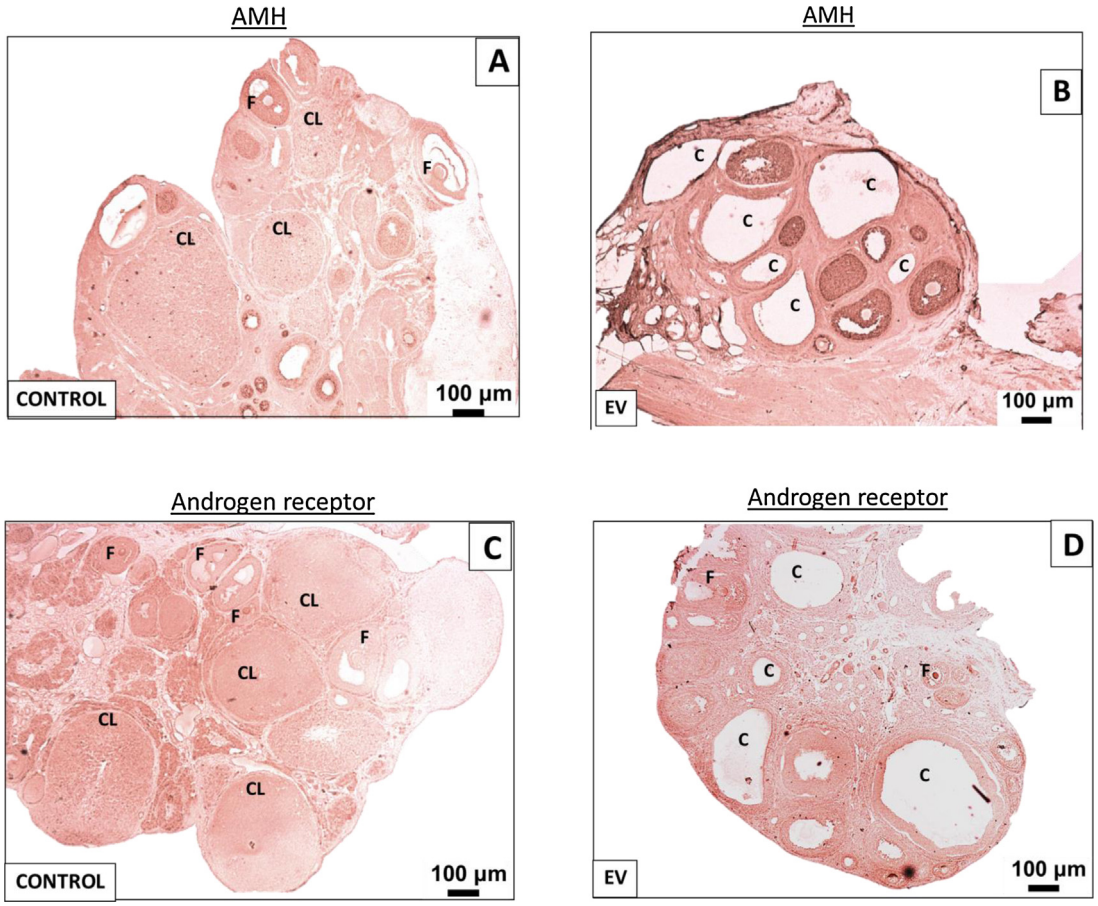

Fig. 4. Gross morphology of the ovaries from adult rats exposed neonatally to a single dose of estradiol valerate (EV). The slices shown are the same in which AMH and AR detection was performed by immunohistochemistry. A and B.- to AMH and C and D to androgen receptor in 60 days old rat ovaries. B and D are representative images from EV-treated rats and A and C from control 60 days old rats. C = cysts, F = antral follicles and CL = corpora lutea. The bar corresponds to 100 µm.

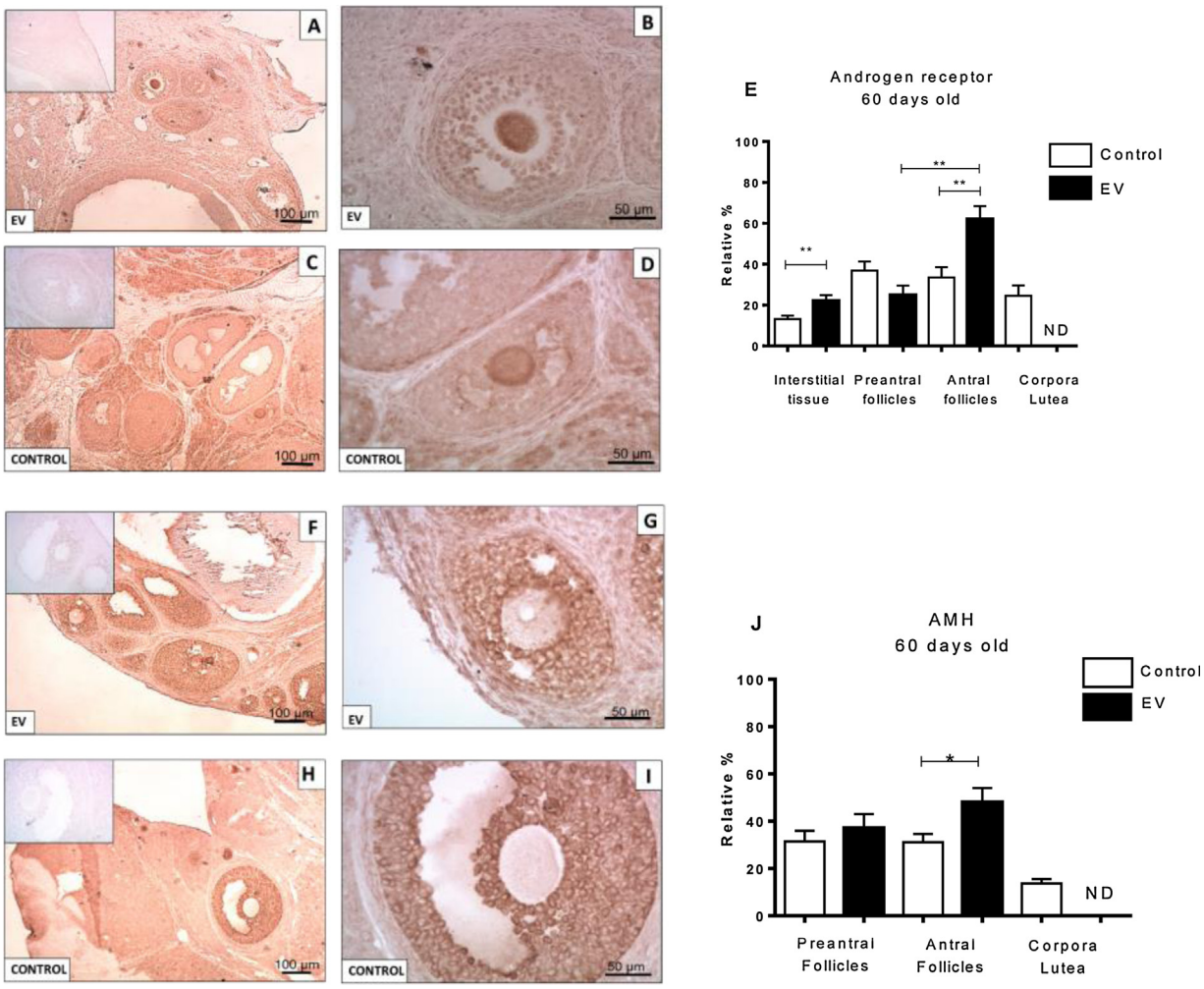

Fig. 5. Distribution pattern of androgen receptor (AR) and anti-Müllerian hormone (AMH) changes due to estradiol exposure in 60 days of age rat ovaries. Immunohistochemical detection of A–D.- androgen receptor and F–I.- AMH in 60 days of age rat ovaries. A-B and F–G are representative images from EV-treated rats and C–D and H–I form control rats. Semiquantification of E.- androgen receptor and J.- AMH, expressed as a relative percentage. The total integrated optical density (IOD) of the whole ovary slice in the image was considered 100% and that against the contribution of each oocyte was calculated. ND means that there is no corpora lutea in the ovaries. Similar sections of at least 4 slides per ovary and 6 ovaries per group were analyzed. Each age group comprised 6 controls and 6 EV female rats. The data are shown as the means ± SEM. * $p < 0.05$ vs control.
** $p < 0.01$ vs control.

tor, and hepatocyte growth factor have previously been found to be altered in the PCO [29–34].

Members of the TGFβ family such as AMH, TGF-β, and the BMPs play important roles in follicle recruitment, follicle selection, and FSH responsiveness [34]. Because of the close relationship between AMH and PCOS in humans, we selected this hormone for the analysis. Using real-time PCR, individual samples were analyzed for the expression pattern through development (2, 30, and 60 days of age rat ovaries). In adult EV-exposed rats (60 days of age), the *Amh* mRNA level was 3.96-fold higher than that of the controls, similar to the results found with the PCR array. In 30 days of age EV-exposed rat ovaries, the *Amh* mRNA level was 2.18-fold higher than that in the controls. There was almost no expression of *Amh* in the ovaries from the 2 days of age EV-exposed and control rats (Fig. 3B).

3.3. EV exposure changes the expression pattern of androgen receptor and anti-Müllerian hormone in the rat ovary

We performed semiquantitative IHC for AR and AMH to determine the expression pattern of these proteins in different follicle types of 60 days of age rat ovaries. Fig. 4 shows the gross morphology of ovaries from control and EV-treated rats. Neonatal exposure to EV produced an anovulatory condition in adults (no corpora lutea) and the appearance of cystic follicles (Fig. 4B and D). The control ovaries present multiple corpora lutea and no cystic follicles (Fig. 4A and C).

Fig. 5A–D shows the IHC results for AR in the 60 days of age rat ovaries, where AR was expressed in the theca, granulosa cells (GCs), oocytes, and interstitial tissue. There was an increased immunoreactive signal in the antral follicles and interstitial tissue from the EV-treated rats compared with that in the control rats, but there were no differences in the preantral follicles between the two groups. In addition, the AR immunoreactivity signal was stronger in the antral follicles than in the preantral follicles in the EV-treated rats (Fig. 5E).

Regarding AMH, a specific immunoreactive signal was observed in the GCs from the primary to antral follicles, but not in the theca cells or interstitial tissue. The AMH immunoreactive intensity was higher in the antral follicles of the treated rats than in their preantral follicles (Fig. 5F-I). When the IOD was normalized, the

preantral and antral follicles from the EV-treated rats had elevated AMH immunoreactivity relative to the controls (Fig. 5J).

4. Discussion

We found evidence that a single exposure to estradiol at levels higher than the normal plasma levels presented during early development induced permanent changes in ovarian morphology and function. This model has been widely used to study PCO and the metabolic effects of hormone expression that resemble human PCOS such as oligo/anovulation, follicular cysts, and insulin resistance when it is administered during the adult stage of development [12–23]. We have previously reported that the exposure of newborn rats to a single dose of EV established the polycystic-ovary condition of adulthood by inducing irreversible damage in folliculogenesis, ovulation, and reproductive physiology [13,16,17], demonstrating that the neonatal stage in rats is a temporal window of sensitivity to the estradiol effects.

Folliculogenesis in rats is initiated during the neonatal period; however, in humans, this process occurs during the third trimester of pregnancy. In other words, the model of neonatal exposure to estradiol mimics or resembles the effect of abnormal estradiol exposure during the gestational period in humans. This is a very important observation because, based on clinical trials and animal models, it has been postulated that PCOS originates during the gestational period [35–37]. Therefore, the rat model is useful to determine the effects of estrogenic compounds without the influence or participation of maternal hormones that could interfere with proper analysis. The use of estradiol valerate, rather than other endocrine disruptor chemicals, has the advantage of a pure estrogenic effect, unlike other animal models that study the effects of endocrine disruptors on the reproductive system. For example, methoxychlor has estrogenic, antiestrogenic, and antiandrogenic activities, while bisphenol-A (BPA), has estrogenic, antiestrogenic, androgenic and thyroid hormone activities [38,39]. Probably, the dose used is very high compared with the concentration used in women using transdermal estradiol treatment to suppress serum gonadotropin secretion during lactation. The single dose of EV applied in our work peaked to 1500 ng/L, indicating that, after 48 h, we obtained 5 times the concentration reached in a chronic implant in women [40–42]. Because the half-life data found in our work was 1.8 days, the effect was not a chronic exposure; thus, it was more dependent on the specific window in which the high levels of estradiol were found. In fact, we previously described that there are specific windows of development in which estradiol can reprogram the ovary function [13]. It could be interesting to follow the female progeny of women who used the estradiol patch during lactation to determine whether there is a reprogramming of ovary function in adults similar to that demonstrated in the daughters of mothers with PCOS [10,43].

In the present study, we found a 13.5-fold increase in the plasma estradiol levels within 24 h after EV exposure. In control newborn Sprague-Dawley rats, the estrogen levels are remarkably high, decaying 48 h after birth [44]. These levels are lower than those observed in EV-administered rats 24 h after exposure, coinciding with the beginning of primordial follicle assembly [3,45]. Placental alpha-fetoprotein binds estradiol and, thus, regulates the availability of circulating free estradiol to the fetus, blocking its effects [44]. However, this protective mechanism cannot buffer estradiol plasma levels higher than those found in fetuses and newborns [44], such as the concentrations found in this study 24 h after the EV-exposure. Our data on the half-life of estradiol allows us to determine that the injected estradiol would last ten days in circulation. Thus, the effects we find are not due to estradiol remaining in the circulation, particularly because, in the plasma of 15 days of age rats, the estradiol levels were the same in the EV and controls groups. Thus, the effects we see are triggered during the neonatal period.

4.1. *Effect of neonatal EV exposure on* Ar *expression in the ovary*

The present data confirm and extend previous studies indicating that female rats exposed to a single dose of estradiol during the neonatal period have permanent modifications to their ovarian morphology and function, presenting a polycystic ovary morphology and infertility [13].

Several researchers have postulated that if estrogen levels remain high postnatally, nest breakdown, follicular assembly, the expression of transcription factors that regulate oocyte development and folliculogenesis, and other growth factors are reduced [4,5,16,17,45]. In our study, the effect of estradiol was also evident at the molecular level beginning 24 h after EV exposure, with increased ovarian mRNA levels of nerve growth factor and p75 low-affinity neurotrophic receptor [16]. The increased expression of AR in the current study was statistically significant beginning 24 h after EV exposure, a finding similar to that found in other genes by Sotomayor-Zarate et al. [16]. The increase in the *Ar* expression might be induced directly by estradiol through the estrogen response elements found in the gene that can regulate the expression in response to estradiol induction [46,47]. Although estradiol plasma levels in 60 days of age rats were comparable between the groups, the *Ar* mRNA levels were diminished (6.4-fold) in EV-treated rats. It is possible that the decreased levels of *Ar* found in the ovaries from EV-treated rats could be due to the decrease in the number of ovarian follicles that normally express *Ar* [16,17]. However, according to IHC results, AR expression was higher in the antral follicles at 60 days in the EV-treatment group. This is an interesting finding because the number of antral follicles is decreased 60 days after EV exposure [13,16], suggesting that the increase in AR signal is not due to the change in the number of follicles but rather to the overexpression of AR by individual follicles. These data imply a failure in the control of AR expression in the antral follicles.

The increased AR expression found in the GC of the antral follicles has been described in testosterone-treated adult monkeys from the preantral to the large antral follicle stage [48,49]. [SG Hillier, M Tetsuka and HM Fraser [50]] have shown that GCs from immature follicles have 4 times as many ARs than GCs from pre-ovulatory follicles, suggesting that this decrease is needed for ovulation. Human studies have also shown a relationship between the increase in AR expression in granulosa cells from PCO patients and the increase in circulating androgens [49]. Thus, this relationship obtained in the neonatal EV-exposed rat is important to understand the underlying mechanisms of the PCOS phenotype.

4.2. *Effect of neonatal EV exposure on the expression of anti-Müllerian hormone in the ovary*

In humans, AMH is expressed by Sertoli cells from the eighth week of gestation until the onset of puberty in males. However, in females, AMH is expressed in granulosa cells and is secreted into the circulatory system from the time of birth until menopause [51] [52], being expressed by primary follicles and continuing to be expressed in follicles in the antral stage. The highest level of AMH expression is present in granulosa cells of secondary, preantral and small antral follicles (<4 mm in diameter). There is no detection of AMH in larger (>4 mm) antral follicles [53]. The serum levels of AMH in normo-ovulatory women decrease over time, so it can be used as a marker of ovarian reserve and aging. Plasmatic levels of AMH seem to correlate with the development of the preantral and small antral follicles, thus making it a marker of ovarian reserve, and it is useful to estimate the reproductive lifespan of healthy

young women [52,54,55]. Women with PCOS have two- to three-fold higher plasma levels of AMH than women with normal ovaries [56]. Additional studies have shown that infants, young, and adolescent daughters of women with PCOS also exhibit higher levels of AMH than control girls [9,10,43].

Our study shows higher immunoreactivity to AMH in the ovary of EV-treated rats, suggesting reprogramming of the expression in GCs induced by the hyper-estrogenic neonatal environment. That there are no granulosa cells during the treatment window raises the question of how GC reprogramming could occur. Although there are no GCs in the first days after birth [3,57], the effects of estradiol could be exerted in precursor cells, as has been shown in a model of prenatal testosterone excess in sheep where exposure causes structural changes [58], in cells that have been proposed as a source of granulosa cells [57]. However, the proposed mechanism is a simplified view of the possible control of AMH through estradiol.

Some studies have shown that the high expression of AMH found in the granulosa cells from PCO patients can inhibit the synthesis of FSHR, and, thus, decrease the response to FSH. During normal follicle development in humans, the AMH levels are higher in the small antral follicles with the levels gradually decreasing as follicle size increases and fall exponentially when the follicles reach 10 mm, the size at which follicle selection occurs. As the follicle size increases and production of AMH declines, the follicles become responsive to FSH and selection can occur [30,59]. The overexpression found in our model may be related to a reduced FSH sensitivity due to a decrease in FSHR expression and aromatase expression and activity, as has been reported in human GCs [59]. This reduction in FSH sensitivity could contribute to the anovulation previously reported as a consequence of the neonatal exposure of estradiol [13,16,17].

5. Conclusions

Understanding these phenomes requires advanced fetal technologies and postnatal assessment of midgestational steroids exposure that can affect the methylation of some specific genes, which can also trigger pathologies during adulthood such as PCOS. Our results with estradiol exposure strongly suggest that the rat ovary is a good experimental model to study the early stages of development and role of epigenetics modifications and their permanent effect of programming effect of high levels of estrogens or other steroids. Worldwide, steroid estrogens, including estrone, estradiol and estriol, pose serious threats to soil, plants, water resources and humans. Indeed, estrogens have gained notable attention in recent years due to their rapidly increasing concentrations in soil and water all over the world.

Author's roles

JM-P performed most of the experimental work with rats and gene arrays. BP conducted the immunohistochemical analyses and, together with J.M.P, analyzed the results. J.M.P and BP contributed equally in the work. MT performed the half-life analysis of EV in neonatal rats as part of her Mg. Sci. Thesis. HL conceived the study, directed the work and, in collaboration with BP, drafted the paper. All authors participated in the writing of the manuscript and approved of the final version.

Conflict of interests

The authors declare that there are no competing financial interests.

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