Temporal window in which exposure to estradiol permanently modifies ovarian function causing polycystic ovary morphology in rats

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Objective: To investigate the developmental window in which E_2 exposure produces irreversible changes in ovarian function resulting in polycystic ovary.
Design: Basic experimental study.
Setting: University animal laboratory.
Animal(s): Thirty Sprague-Dawley rats were administered a single E_2 valerate dose (10 mg/kg of weight) at 1, 7, 14, 21, or 30 days of age. Control rats were injected with the vehicle at 1 day of age. All rats were sacrificed at 6 months of age.
Intervention(s): Observation of vaginal opening, estrous cyclicity by vaginal smears, and ovarian morphometry in the 6-month-old rat.
Main Outcome Measure(s): Measurement of ovarian noradrenaline by high-performance liquid chromatography coupled with electrochemical detection, serum levels of LH by enzyme-linked immunoassay, P, androstenedione, and E_2 by enzyme immunoassay.
Result(s): Rats exposed to E_2 at 1, 7, or 14 days of life did not show estrual cycling activity and maintained a polycystic ovary (PCO) condition throughout the entirety of the study. However, if the exposure to E_2 occurred after postnatal day 21, the PCO-induced condition was reversible. In rats that developed a permanent PCO condition, we observed significant effects of E_2 on ovarian morphology if exposure occurred on postnatal day 1 and a presumable effect on the hypothalamus if the exposure occurred between postnatal days 1 and 14.
Conclusion(s): Our findings suggest that in rats, the most sensitive period for the promotion of an irreversible PCO morphology by estrogenic compounds is during neonatal early follicular development. (Fertil Steril® 2012;98:1283–90. ©2012 by American Society for Reproductive Medicine.)
Key Words: Estradiol, ovary, follicular development, neonatal programming

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Exposure to natural or environmental estrogenic compounds in early life disrupts ovarian function and hypothalamic activity in various species (1–3). The administration of a single dose of E_2 valerate (EV) to rats at different ages before puberty promotes the development of an anovulatory condition characterized by impaired follicular development and the presence of follicular cysts in adult rats (4–6). The fact that these effects could be provoked by a single administration of the steroid during a specific window of postnatal development evokes the concept of "programming," which is defined in a physiological context as an early stimulus or insult during a critical hormone-sensitive period that results in negative effects during adulthood (7). To obtain evidence for this concept, it is necessary to determine whether the effect is permanently expressed during the life span.

Polycystic ovarian syndrome (PCOS) is a complex endocrine disorder characterized by hyperandrogenism,

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chronic anovulation, and polycystic ovaries (PCO) (8, 9). Several studies in animal models have demonstrated a relationship between prenatal or neonatal exposure to endocrine-disrupting compounds, such as estrogens or aromatizable androgens, and programmed PCO morphology during adulthood (1–5). The early observation that pregnant women with PCOS present enlarged ovaries (10) and higher levels of serum androgens during pregnancy (11) as well as studies of early hormone exposure and programming of reproductive function in various animal models have led to the hypothesis that PCOS originates during development (12–14). In addition, human studies have suggested that PCO morphology and menstrual disorders are persistent conditions in women with PCOS throughout their reproductive years, even up to menopause (15–17). These studies lead to the concept that changes in programming, which might be epigenetic, persist during development and could be responsible for the phenotypic characteristics of this syndrome. Animal models representing this lifelong persistence are difficult to obtain. Owing to its relatively short life cycle and early neonatal development of follicles, the rat could be a useful model to follow the early changes of follicular development that result in PCO during adulthood. Thus, in this study we looked for the specific developmental time window in which E_2 exposure generates an irreversible PCO morphology and estrual abnormalities that persist up until the perimenopausal period of development.

MATERIALS AND METHODS

Animals

Thirty Sprague-Dawley rats derived from a colony maintained at the University of Chile were used in this study. EV (10 mg/kg of weight) was SC administered to rats at 1, 7, 14, 21, and 30 days of age, and control rats were injected with vehicle only (sesame oil) at postnatal day 1. The volume of EV injection was 0.05 mL for postnatal day 1 and 0.1 mL for other experimental groups.

Because rats treated with EV at different ages are in persistent estrous (4, 5, 18, 19), control rats were also killed during the estrus stage of the cycle (between 9:00 and 12:00 A.M.). Control and EV-treated rats were killed at 6 months old because at this age control rats are still completely fertile and ovarian aging has not yet begun.

All experimental procedures were approved by the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences at the Universidad de Chile and complied with national guidelines (CONICYT Guide for the Care and Use of Laboratory Animals). All efforts were made to minimize the number of animals used and their suffering.

Morphology

One ovary from each control or EV-treated rat was immersed in Bouin's fixative, embedded in paraffin, cut into 6 μm sections, and stained with hematoxylin and eosin. For morphometric analysis, a previously reported criterion was used. All follicular structures were followed through all slices and were counted when they reached the largest diameter. One exception was primordial follicles, which were counted every three slices to avoid overcounting. The follicles were classified according to their histological characteristics. Briefly, primordial follicles were those with one oocyte surrounded by flattened granulose cells; primary follicles were counted as primordial follicles exhibiting one layer of cubical granulose cells; secondary follicles had no antral cavity but did have two or more layers of granulose cells; atretic follicles had more than 5% of cells with pyknotic nuclei in the largest cross-section and exhibited shrinkage and an occasional breakdown of the germinal vesicle; antral follicles were those in which the nucleus of the oocyte could be visualized; type III follicles were large follicles containing four or five plicated layers of small, densely packed granulose cells surrounding a very large antrum with an apparently normal thecal compartment; finally, cystic follicles were devoid of oocytes and displayed a large antral cavity, a well-defined thecal cell layer, and a thin (mostly monolayer) granulose cell compartment containing apparently healthy cells (18). All abnormal follicular structures were grouped as cystic structures.

Determination of Serum Levels of LH, E_2, Androstenedione, and P

All hormone determinations of blood samples obtained at the end of the study (6-month-old rats) were performed. LH levels were determined by enzyme-linked immunoassay according to the manufacturer's instructions (Repropharm). The coefficient of variation (CV) was less than 10%, and the sensitivity was 0.2 ng/mL.

E_2, androstenedione, and P levels were determined by enzyme immunoassay according to the manufacturer's instructions (Alpco Diagnostic). Intra- and interassay variations were less than 5% for E_2, less than 6% for androstenedione, and less than 5% for P. The minimal detectable values were 10 pg/mL, 0.05 ng/mL, and 0.1 ng/mL, respectively.

Determination of Ovarian Levels of Norepinephrine by High-Performance Liquid Chromatography (HPLC)

Ovaries were collected on dry ice and stored at $-80°C$. For the assay, the ovary was weighed, homogenized (perchloric acid; 0.25 N), and centrifuged at 12,000 $\times$ g for 10 minutes at 4°C. The resulting supernatant was filtered and used for measurements. A total of 20 μL of each filtrate solution was loaded into an HPLC system as described elsewhere (5, 20). Samples were analyzed by comparing their peak area and elution times with those of reference standards.

Statistical Analysis

Data are expressed as the mean $\pm$ SEM. To determine significant differences among time points or multiple groups, we used one-way analysis of variance followed by the Newman-Keuls post hoc test.