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Differences in neonatal exposure to estradiol or testosterone on ovarian function and hormonal levels

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ABSTRACT

Exposure to an excess of androgen or estrogen can induce changes in reproductive function in adult animals that resemble polycystic ovary syndrome in humans. However, considerable differences exist among several types of animal models. Little is known about the molecular features of steroidogenesis and folliculogenesis in the ovaries of rats exposed to different sex steroids as neonates. Here, we evaluated the impact of androgen and estrogen exposure on the ovaries of adult female rats during their neonatal period in the gene expression of *Lhr* and *Cyp17a1*, two key players of steroidogenesis. We also assessed hormone levels, folliculogenesis and the theca-interstitial cell population. The study was performed on the second postnatal day in thirty female *Wistar* rats that were sorted into the following three intervention groups: testosterone, estradiol and vehicle (control group). The animals were euthanized 90 days after birth. The main outcomes were hormone serum levels, ovary histomorphometry and gene expression of *Lhr* and *Cyp17a1* as analyzed via quantitative real-time PCR. We found that exposure to excess testosterone in early life increased the LH and testosterone serum levels, the LH/FSH ratio, ovarian theca-interstitial area and gene expression of *Lhr* and *Cyp17a1* in adult rats. Estrogen induced an increase in the ovarian theca-interstitial area, the secondary follicle population and gene expression of *Lhr* and *Cyp17a1*. All animals exposed to the sex steroids presented with closed vaginas. Our data suggest that testosterone resulted in more pronounced reproductive changes than did estrogen exposure. Our results might provide some insight into the role of different hormones on reproductive development and on the heterogeneity of clinical manifestations of conditions such as polycystic ovary syndrome.

1. Introduction

Exposure to excessive androgen or estrogen can induce changes in the reproductive functions of several animal models that resemble polycystic ovary syndrome (PCOS) in humans (Franks, 2012). A growing body of evidence has suggested that several metabolic conditions may be related to the fetal period or early life events (Barker, 1990, 1993; Franks, 2012). Genetic predisposition and environmental insults, such as maternal obesity, diet and exposure to several substances, including sex steroids (Franks, 2012), could trigger some specific metabolic phenotypes that might manifest during adulthood. In this sense, PCOS has gained attention because it displays reproductive function abnormalities (e.g., anovulation and disordered ovarian follicle development) and metabolic traits (insulin resistance, metabolic syndrome and obesity) beginning in puberty that seems to fit this hypothesis (Legro et al., 1998; Vink et al., 2006; Franks et al., 2008b; Chen et al., 2011). However, because this hypothesis is difficult to prove in humans, animal models might be invaluable in the exploration of this issue.

PCOS is an endocrine disorder characterized by hyperandrogenism and chronic anovulation and is related to impairment in the hypothalamic–pituitary–ovarian axis and ovarian abnormalities (Baptiste et al., 2010). LH levels are elevated in 40–50% of patients with PCOS (Homburg, 2008), probably due to hypothalamic dysfunction (Pastor et al., 1998). Higher levels of LH lead to an increased androgen production by the theca cells (Chang, 2007; Magoffin, 2006). Additionally, overexpression of cytochrome P450c17 (*CYP17*), a key steroidogenic enzyme, has been shown to convert pregnenolone and progesterone into dehydroepiandrosterone (DHEA) and androstenedione, respectively (Magoffin, 2006;

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Baptiste et al., 2010). Furthermore, PCOS ovaries display an hyperplasia of steroidogenic cells in the follicles and in the ovarian stroma as well as disordered ovarian folliculogenesis (Chang and Cook-Andersen, 2013) that include a global increase in the number of follicles, a higher percentage of early growing (primary) follicles and a greater number of growing follicles at each stage of development (Hughesdon, 1982; Webber et al., 2003; Maciel et al., 2004; Franks et al., 2008a). However, a great degree of heterogeneity exists in the clinical and metabolic features of PCOS, and the reasons for this heterogeneity are unknown.

Several models have been developed in an attempt to understand the potential contribution of exposure to excessive steroids on chronic anovulation and abnormal production of reproductive hormones, such as exposure to androgens (testosterone, dihydrotestosterone, dehydroepiandrosterone sulfate), estrogens, and aromatase inhibitors, among others (Padmanabhan and Veiga-Lopez, 2013). Previous studies have shown that androgen interferes with the ovulation process and might have a direct effect on the ovary (Sjaarda et al., 2014). On the other hand, estrogens are also able to induce anovulation and disrupt reproductive functions, but how it affects specific steps of the reproductive physiology is not well known (Alexanderson et al., 2010; Franks, 2012).

However, it is not known which sex steroid, testosterone or estradiol, induces the most intense functional and structural ovarian changes that closely mimic PCOS. In this study, we investigated the effects of exposure to testosterone or estradiol in early life on the following: (1) gene expression of key players of steroidogenesis in the ovary of adult female rats, especially *Lhr* (luteinizing hormone receptor) and *Cyp17a1* (cytochrome P450C17); (2) circulating hormonal levels; and (3) ovarian morphology. The rationale is to investigate whether exposure to different steroids leads to different phenotypes that mimic PCOS heterogeneity.

2. Materials and methods

This study was approved by the Institutional Ethics Committee of the Faculdade de Medicina da Universidade de São Paulo (CEP-FMUSP) under protocol number 151/10 and complies with the Brazilian and International rules for scientific use of animals (Marques et al., 2009).

2.1. Animals

Thirty female Wistar rats (*Rattus norvegicus albinus*) aged between 0 and 2 days were used in this study and were housed at the Centro de Bioterismo da Faculdade de Medicina da Universidade de São Paulo. The thirty female pups were sorted into five per cage with their respective dams. Dams were maintained with their pups until weaning (21 days). The animals were maintained at a temperature of 22 °C under artificial illumination on a light–dark cycle of 12:12 h, with daylight from 7 a.m. to 7 p.m until the sacrifice day. Food and water were given *ad libitum*.

2.2. Treatment protocols

Thirty rats in the second postnatal day were sorted into three experimental groups containing ten animals each. The division was performed according to the single subcutaneous administration of the following compounds: 1.25 mg of testosterone propionate (testosterone group, TG; $n = 10$) diluted in 0.1 mL of olive oil (vehicle) (Mahamed et al., 2011), 0.5 mg of estradiol benzoate (estradiol group, EG; $n = 10$) diluted in 0.1 mL of vehicle (Alexanderson et al., 2007) and vehicle only (control group, CG; $n = 10$).

2.3. Estrous cycle evaluation and euthanasia

Vaginal smears were collected on glass slides to evaluate the animals' estrous cycles from 75 days to 90 days of age. Shorr–Harris staining was used to analyze the vaginal cytology. A normal estrous cycle was defined as exhibiting all phases (proestrus, estrus, metestrus, and diestrus) over a period of 4–5 days. At 90–94 days of age, the animals were transferred to the surgery room of the *Centro de Bioterismo da Faculdade de Medicina da Universidade de São Paulo* and then anesthetized with ketamine (50 mg/kg)/xilazyne (5 mg/kg) intraperitoneally. Between 11:00 a.m. and 13:00 p.m., blood samples were collected, and the ovaries were removed before the animals were euthanized. All of the control animals were euthanized in the estrous phase of the cycle.

2.4. Analysis of the serum levels of LH, FSH and testosterone by enzyme-linked immunosorbent assay (ELISA)

Blood samples were centrifuged at 4 °C and 5000 rpm/4696×*g* (Sorvall – Thermo Scientific, Asheville, NC, United States) for 15 min to separate the blood's solid components from the serum. Then, the serum was collected and stored at −20 °C until quantification of the LH, FSH and total testosterone levels using ELISA kits (Ucsn Life Sciences Inc., Wuhan, Hubei, China). The lower level of detection for LH, FSH and testosterone kits was 0.13 ng/mL, 0.92 ng/mL, and 0.05 ng/mL, respectively. For all kits, the intra-assay and inter-assay coefficient of the variations were <10% and <12%, respectively.

2.5. Morphological and morphometric analyses of the ovaries

The ovaries were fixed in 10% buffered formaldehyde for 24 h. They were then submitted to the following steps: ethyl alcohol dehydration, diaphanization in xylene, liquid paraffin impregnation in a drying stove at 60 °C, and paraffin inclusion at room temperature.

The paraffin blocks containing the tissues were sectioned using a microtome (Microm HM315 R, MICROM International GmbH, Walldorf, Baden-Württemberg, Germany), adjusted for sections of 4 μm thickness on glass slides. Afterward, each slide was stained with Hematoxylin and Eosin according to classic protocols. The morphological and morphometric analyses were conducted using an optical microscope (Leica, Wetzlar, Hesse, Germany) coupled with a digital camera system (Leica DFC420).

The morphometric analysis was performed separately by two observers using the Leica Application Suite 3.0.0. software. Three sequential sections, at least 100 μm apart from the inner third of each ovary, were used to count the follicles and corpora lutea and to quantify theca-interstitial area (adapted from Regan et al., 2005).

Counting the follicles and *corpus luteum* was performed by 10 photomicrographs at 200× magnification taken from different fields of each slide. The criteria for follicle counting were as defined by Maciel et al., 2004.

To quantify the ovarian theca-interstitial area, we first measured the total cross-sectional area at 50× magnification and then the interstitial area at 100× magnification. The theca-interstitial area-to-total cross-section area ratio was calculated on each slide using the formula x = theca-interstitial area with a 100/total cross-section area (Lombardi et al., 2012).

2.6. Total RNA extraction

Ovarian total RNA extraction was performed using organic extraction methods (TRIzol Reagent, Life technologies, Foster City, CA, United States) following the manufacturer's instructions. The